Swapping between Fas and Granulocyte Colony-stimulating Factor Receptor*

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G-CSFR is a member of the hemopoietic growth factor receptor family. G-CSF induces its dimerization and regulates the proliferation and differentiation of neutrophilic granulocytes. We constructed hybrid receptors between Fas and G-CSFR and expressed them in the mouse T cell line WR19L or the mouse myeloid interleukin-3-dependent FDC-P1 cell line. The Fas ligand or an agonistic anti-Fas antibody stimulated proliferation of the FDC-P1 transformants expressing a chimera consisting of the Fas extracellular and G-CSFR cytoplasmic regions. On the other hand, G-CSF could not induce apoptosis in the transformants expressing the chimera consisting of the G-CSFR extracellular and Fas cytoplasmic regions, but these cells were killed by a polyclonal antibody against G-CSFR. These results indicated that receptors belonging to different receptor families can be functionally exchanged and confirm that a homodimer of G-CSFR can transduce the growth signal, whereas Fas must be oligomerized (probably trimerized) to transduce the apoptotic signal.

Cellular proliferation, differentiation, and apoptosis are regulated by protein factors, collectively called cytokines. Cytokines are grouped into at least three subfamilies based on structure, cystine knot growth factors, tumor necrosis factor (TNF) and helical cytokines (1). G-CSF (granulocyte colony-stimulating factor) is a 20-kDa, helical glycoprotein cytokine, and it belongs to the hemopoietic growth factor family (2). G-CSF binds to its specific receptor and stimulates the proliferation and differentiation of neutrophilic progenitor cells. The G-CSF receptor (G-CSFR) is a type I membrane protein belonging to the hemopoietic growth factor receptor family (3, 4), which includes the receptors for interleukins (ILs), colony-stimulating factors (CSFs), and growth hormone. Most members of the hemopoietic growth factor receptor family consist of two different subunits, and a heterodimer binds the cognate ligand with high affinity, thus transducing the signal. G-CSFR, the receptors for erythropoietin and growth hormone are made of a single polypeptide, and the ligand seems to induce homodimerization of the receptor, which transduces the signal (2). Exogenous expression of G-CSFR in an IL3-dependent myeloid precursor such as FDC-P1 makes the cells respond to G-CSF by proliferation (5). Mutational analyses of G-CSFR have indicated that a domain of about 200 amino acids in the extracellular region of G-CSFR, which shows a similarity with other hemopoietic growth factor receptors, is the G-CSF binding site (5).

Fas, also called APO-1 or CD95, is a 45-kDa type I membrane protein expressed in various tissues and cells and belongs to the TNF/nerve growth factor (NGF) receptor family (6–9). This family is comprised of TNF receptors (type I, β or 55 kDa; type II, α or 75 kDa), low affinity NGF receptor, the B cell antigen CD40, as well as the T cell antigens CD27, CD30, OX40, and 4-1BB. Most ligands of the TNF/NGF receptor family belong to the TNF family, which includes TNF, lymphotoxin, Fas ligand (FasL), CD40 ligand, etc. (8, 10). FasL is synthesized as a type II membrane protein with a molecular mass of 40 kDa (11), and under certain conditions, a trimeric soluble form of FasL is produced by proteolytic cleavage (12, 13). The membrane-bound form of FasL, the soluble form of human FasL, or the agonistic antibodies against Fas induce apoptosis by binding to Fas in Fas-bearing cells (6, 11, 12, 14, 15).

Hybrids between members within the hemopoietic growth factor receptor or TNF/NGF receptor families bind the ligand corresponding to the extracellular region of the hybrid and transduce the signal specific to the cytoplasmic region of the chimeric receptor (16, 17). In this study, we constructed hybrids between G-CSFR and Fas, which belong to different cytokine receptor families (hemopoietic growth factor and TNF/NGF receptor families). The chimeric receptor carrying the cytoplasmic region of G-CSFR transduced the growth signal, whereas carrying the Fas cytokimic region transduced the apoptotic signal when they dimerized or oligomerized, respectively. These results indicated that the role of the ligand is just to induce dimerization or oligomerization of the respective receptors to activate them.

MATERIALS AND METHODS

Construction of Expression Plasmids—The expression plasmids for chimeric receptors between Fas and G-CSFR were constructed by reconstituent polymerase chain reaction (PCR) essentially as described.
The following primers were prepared using a DNA synthesizer (Applied Biosystems model 370A). MFB (AGGTACTAATAGCATCTCCG) and MOR3 (ACCTGGACAGCCTGAGG) were forward primers corresponding to the sequences of mouse Fas or mouse G-CSFR cDNA. The following two primers carried hybrid sequences consisting of the antisense strand of mouse Fas and the G-CSFR: MFB (TCCAGCTCTAGGTTTGGGGCTAT), and MOR3 (ACCTGTCTGGAGGTGCTAT). MFBGT1, CACATGGGATTTGGATCTAGCTCTTCAAGGGTGTAAGG. MFMRG15 and MFGR1 primers were complementary to MFMGR1A and MFGR1A, respectively. The MOR12 (GGACCTTCGTTTCAAGGAA) and MFRF (AGCAGGTGAGTACATT) primers carried the antisense sequences of mouse G-CSFR and Fas.

PCR for pBOS-EA, carrying the coding sequence for mouse Fas (18), and pBOS-162, carrying the full-length cDNA for mouse G-CSFR (5), were as described. To construct the expression plasmid for the Fas/G chimera carrying the Fas extracellular and G-CSF cytoplasmic domains, part of the Fas cDNA in pBOS-EA was amplified using MFB and MFGR1A primers, and part of the G-CSFR cDNA in pBOS-162 was amplified using MFMRG15 and MOR12 primers. The conditions for PCR were 1 min at 94 °C, 2 min at 55 °C, 3 min at 72 °C for 20 cycles. The PCR products were isolated by agarose gel electrophoresis and treated with the Klenow fragment of DNA polymerase I. These products were mixed 1:1, and a second PCR proceeded as described above using MFB and MOR12 primers. The product from second PCR was digested with HindIII and PstI and ligated in three ways with the DNA fragment from pBOS-EA digested with HindIII and SplI, and the fragment from pBOS-162 digested with PstI and SplI. The expression plasmid for the other chimeric receptor, G/Fas, which is a hybrid between the mouse G-CSFR extracellular and Fas cytoplasmic domains, was constructed similarly, using MFGTS1 and MFFR primers for the first PCR and MOR3 and MFFR primers for the second. The product from the second PCR was digested with KpnI and HindIII and ligated with DNA fragments of the appropriate 5’ and 3’ portions of G-CSFR and Fas cDNA, respectively.

Antibodies and Cytokines—Hamster anti-mouse Fas monoclonal antibody (clone J02) was as described (18). To produce other anti-Fas monoclonal antibodies, rats were immunized with the soluble form of mouse Fas (M-Fas-Fc), and the spleen cells from the immunized mice were fused with mouse myeloma P3X63Ag8A. A hybridoma producing the anti-Fas antibody suitable for Western blotting was selected, and the antibody in the culture supernatant of the hybridoma (OB10) was purified with protein A-Sepharose. The anti-mouse G-CSFR (anti-MR1) recognizing its cytokine receptor-homologous (CRH) domain was as described (5). Human recombinant G-CSFR produced in Chinese hamster ovary cells were provided by Chugai Pharmaceutical Co. Production, and purification of human recombinant FasL will be described elsewhere.2 Biotinylation of the anti-Fas antibody and G-CSF was carried out with NHS-LC-biotin (Pierce) as described (18, 19).

Cells and Transfection—The mouse T cell lymphoma, WR19L (ATCC TIB52) was maintained in RPMI 1640 medium containing 10% fetal calf serum, and 10% fetal calf serum in the myeloid precursor cell line FDC-P1 (20) kindly provided by Dr. T. M. Dexter was cultured in RPMI 1640 medium containing 10% fetal calf serum and 75 units of recombinant mouse IL-3/ml. WR19L and FDC-P1 cells were transfected with the expression plasmid by electroporation as described (5, 6). After selection with G418 (0.9 mg/ml WR19L and 8.5 mg/ml FDC-P1), transformants were analyzed by flow cytometry using a biotinylated mouse anti-Fas antibody (J02) (18) or biotinylated G-CSF (19). The flow cytometry was performed essentially as described previously (18).

Western Blotting—Cells were grown to a density of 1 x 10^6 cells/ml, washed with phosphate-buffered saline, and suspended to 1 x 10^6 cells/ml in lysis buffer (0.5% CHAPS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 2 mM (p-aminophenyl) imethanesulfonyl fluoride, 2 mM EDTA, 2 mM p-phenanthroline, 0.1 mM leupeptin, 1 μg/ml pepstatin A, 100 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, 50 μg/ml soybean serin peptidase, and 50 μg/ml soybean phosphatase, 10 μg/ml sodium orthovanadate, 0 μM sodium fluoride, and 10 μM sodium pyrophosphate). Aliquots of the lysates corresponding to 3.8 x 10^6 cells were resolved by electrophoresis on 4-20% gradient polyacrylamide gels under reducing conditions and then transferred onto a polyvinyl difluoride membrane. Western blotting analysis proceeded using rat anti-Fas monoclonal antibody (clone OB10) or rabbit anti-G-CSF antibody (MR1). Chemical cross-linking of biotinylated G-CSF with the receptor and its recovery with streptavidin-agarose (Molecular Probe, Inc.) were performed as described previously (21).

RESULTS

Construction of cDNAs for Chimeric Receptors Between Fas and G-CSFR—Fas and G-CSFR consist of extracellular, transmembrane, and cytoplasmic regions (Fig. 1). The Fas extracellular region is rich in cysteine residue and can be divided into three subdomains. The extracellular region of G-CSFR has a composite structure consisting of immunoglobulin-like, CRH, and three fibronectin type III domains (22). The CRH domain is responsible for binding G-CSF (5). We constructed two chimeric receptors between Fas and G-CSFR (Fig. 1). In the first Fas/G chimera construct, the Ig-like and CRH domains of G-CSFR were replaced with the extracellular region of Fas. The second chimera, G/Fas, carried the extracellular region of G-CSFR as well as the transmembrane and cytoplasmic regions of Fas. The exon-intron borders of Fas and G-CSFR chromosomal genes (23, 24) were selected as junctions of the chimeras to minimize possible distortion of the structural domain of Fas and G-CSFR. The chimeric receptor cDNAs were constructed by recombinant PCR as described under "Materials and Methods" and placed under the promoter of human elongation factor 1α of the pEF-BOS vector (25).

Expression of Chimeric Receptors—To examine the signal-transducing ability of the Fas/G and G/Fas chimeras, the expression plasmid for Fas/G was introduced into FDC-P1 cells together with the neo-resistant gene, and that of the G/Fas chimera was introduced into WR19L cells. The G418-resistant transformants were then analyzed by flow cytometry using biotinylated anti-Fas antibody for Fas/G and biotinylated G-CSF for G/Fas, respectively. Several FDC-P1 and WR19L transformants were found to bind anti-Fas antibody and biotinylated G-CSF, respectively (data not shown). Three clones for each transformant (FDC-P1-G20, FDC-P1-G29, and FDC-P1-G30 for the FDC-P1 cells expressing the Fas/G chimera; WR-G/Fas-2, WR-G/Fas-6, and WR-G/Fas-9 for the WR19L cells

Fig. 1. Structure of chimeric receptors between Fas and G-CSFR. Open and shaded areas represent domains derived from Fas and G-CSFR, respectively. CR and TM in Fas indicate the cysteine-rich and transmembrane domains. The open box in the Fas cytoplasmic region is the death domain that is homologous with TNF receptor type I. Ig-like, CRH, FNIII, and TM in G-CSFR indicate immunoglobulin-like, cytokine receptor-homologous, fibronectin type III, and transmembrane domains, respectively.

Proliferation and Cell Killing Assay—Cell proliferation and killing were assayed by the MTT procedure as described (12). In brief, 4 x 10^3 cells in 100 μl were cultured for 18–20 h in 96-well plates with various concentrations of G-CSF, Fasl, anti-G-CSFR antibody (MR1) or anti-Fas antibody (J02). Twenty microliters of MTT (2.5 mg/ml) dissolved in phosphate-buffered saline was added to each well and incubated for 4 h at 37 °C. The precipitates were dissolved by adding 150 μl of acid isopropyl alcohol (0.04 N HCl in isopropyl alcohol), and the absorbance was measured using a MicroELISA reader with a test wavelength of 540 nm and a reference wavelength of 690 nm.
designated about 120 kDa could be recognized with the anti-G-CSFR antibody, bands recognized by the anti-Fas antibody, but a protein of the transformants expressing the G/Fas chimera exhibited the recognized with the anti-Fas antibody (Fig. 2).

Wild type mouse Fas showed a band of 45 kDa which can be visualized using the Enhanced Chemiluminescence system (Amersham) after staining with streptavidin-conjugated horseradish peroxidase. Sizes of the marker proteins are indicated on the left in kDa. Lane 1, FDC-P1; lane 2, FD62M expressing the wild type G-CSFR; lane 3, FD-Fas/G-20; lane 4, FD-Fas/G-29; lane 5, FD-Fas/G-30; lane 6, WR19L; lane 7, W4 expressing the wild type Fas; lane 8, WR-G/F-as-2; lane 9, WR-G/F-as-6; lane 10, WR-G/F-as-9.

Lane 2
Lane 3
Lane 4
Lane 5
Lane 6
Lane 7
Lane 8
Lane 9
Lane 10

FIG. 2. Expression of chimeric receptors in transformants. Cell lysates from parental cells and transformants were analyzed by Western blotting using rat anti-Fas antibody (panel a) or rabbit anti-G-CSFR antiserum (panel b). The proteins recognized by the antibodies were visualized using the Enhanced Chemiluminescence system (Amersham) after staining with streptavidin-conjugated horseradish peroxidase. Sizes of the marker proteins are indicated on the left in kDa. Lane 1, FDC-P1; lane 2, FD62M expressing the wild type G-CSFR; lane 3, FD-Fas/G-20; lane 4, FD-Fas/G-29; lane 5, FD-Fas/G-30; lane 6, WR19L; lane 7, W4 expressing the wild type Fas; lane 8, WR-G/F-as-2; lane 9, WR-G/F-as-6; lane 10, WR-G/F-as-9.

The WR19L cell transformant (W4) expressing the wild type Fas and the FDC-P1 transformants expressing the G-CSFR antibody recognizes the extracellular region of Fas, and anti-G-CSFR antibody recognizes the CRH domain in the extracellular region of G-CSFR. The parental FDC-P1 and WR19L cells expressed neither G-CSFR nor Fas (lanes 1 and 6). The clone FD62M expressing the wild type G-CSFR in FDC-P1 cells showed bands of 110–120 kDa (Fig. 2b, lane 2). No corresponding proteins were detected in the FDC-P1 transformants expressing the Fas/G chimera, but they showed a band at 60 kDa (Fig. 2b, lanes 3-5), which seems to be nonspecific. On the other hand, the anti-Fas antibody specifically detected proteins of 95–120 kDa in these transformants (Fig. 2a, lanes 3-5). The apparent molecular mass of the proteins is significantly larger than that expected from the amino acid sequence of the G/F as chimera (88.7 kDa), suggesting that this chimeric protein is also glycosylated.

FasL or Anti-Fas Antibody-induced Proliferation of the FDC-P1 Transformants Expressing the Fas/G Chimeras—The IL-3-dependent FDC-P1 cells do not express G-CSFR. When IL-3 in the culture medium was replaced by G-CSF, the cells died within 10 h (5). On the other hand, the FDC-P1 cell transformants expressing the wild type G-CSFR responded to G-CSF for proliferation (Fig. 3a). The transfectants grew in the presence of 50 ng/ml G-CSF as efficiently as in the presence of 75 units/ml IL-3. Polyclonal antibody against G-CSFR also stimulated proliferation of the transformants but not the parental FDC-P1 cells (Fig. 3b). After incubation, the cell proliferation was determined by MTT assay and is expressed as percentages of that obtained with 75 units/ml IL-3.

Fig. 2. Growth signal transduced by the wild type G-CSFR and Fas/G chimeric receptor. Panels a and b, parental FDC-P1 (closed circles) and FD62M expressing the wild type G-CSFR (open circles) were incubated at 37 °C for 18 h with the indicated concentrations of G-CSF (panel a) or anti-G-CSFR antibody (panel b). The concentration of anti-G-CSFR antibody is expressed as concentrations of the neat serum. Panels c and d, parental FDC-P1 cells (closed circles) or three independent transformant clones expressing the Fas/G chimera (open symbols) were incubated at 37 °C for 20 h with various concentrations of the soluble form of FasL (panel c) or anti-mouse Fas antibody (Jo2) (panel d). After incubation, the cell proliferation was determined by MTT assay and is expressed as percentages of that obtained with 75 units/ml IL-3.
transfectants was significantly higher than that of G-CSF for the wild type G-CSFR, which may be due to some structural distortion of the chimeric receptor.

Anti-G-CSFR Antibody but Not G-CSF Induces Apoptosis in WR19L Transformants Expressing the G/Fas Chimera—Mouse WR19L cells are susceptible to Fas-mediated apoptosis. As shown in Fig. 4, neither FasL nor agonistic anti-Fas antibody caused apoptosis in WR19L cells, but its transformation with the G/Fas chimera expressed in WR19L cells was significantly higher than that of G-CSF for the wild-type G-CSFR. The effect of G-CSF on the WR19L transformants expressing G/Fas chimera was shown in Fig. 4a and b. The G-CSF receptor complex was recovered with streptavidin-agarose beads and analyzed by Western blotting using anti-G-CSFR antibody (closed circles) or anti-G-CSFR antibody (open circles). Cell viability was determined by MTT assay and shown as a percentage of that obtained without FasL or the antibody.

**Fig. 4.** Apoptotic signal transduced by wild type Fas and G/Fas chimeric receptor. Panels a and b, WR19L (closed circles) and transformant W4 expressing wild type Fas (open circles) were cultured at 37 °C for 20 h with various concentrations of the soluble form of FasL (panel a) or anti-Fas antibody (panel b). Panels c and d, parental WR19L cells (closed circles) and its three independent transformant clones (open symbols) were cultured at 37 °C for 18 h with various concentrations of G-CSF (panel c) or anti-G-CSFR antibody (panel d). Cell viability was determined by MTT assay and shown as a percentage of that obtained without FasL or the antibody.

**Fig. 5.** Chemical cross-linking of the G/Fas chimeric receptor with biotinylated G-CSF. WR19L cells expressing G/Fas chimera (1.0 × 10^7 cells) (lanes 1-4) or its parental WR19L cells (lane 5) were incubated for 4 h at 4 °C with 100 ng/ml biotinylated G-CSF in the presence of unlabeled G-CSF. After washing with phosphate-buffered saline, the cells were treated for 10 min on ice with (lanes 1 and 3) or without 0.15 M diisuccinimidyl suberate (lanes 2, 4, and 5) and were lysed with 1% Nonidet P-40. The G-CSF-receptor complex was recovered from the lysates by streptavidin-agarose beads and analyzed by Western blotting using anti-G-CSFR antibody (MR1) as described in the legend to Fig. 2. Sizes of marker proteins are indicated on the left in kDa.

**DISCUSSION**

A single transmembrane region of the cytokine receptor divides the molecule into the extracellular and cytoplasmic regions. The receptor activation due to extracellular ligand binding should be transferred across the membrane barrier to activate the cytoplasmic signal-transducing domain of the receptor. In cytokine receptors containing the tyrosine-kinase domain, the ligand induces dimerization of the receptor, and molecular interaction of the adjacent cytoplasmic domain leads to activation of the kinase (27, 28). Among the hemopoietic growth factor receptors, receptors for G-CSF and growth hormone as a homodimer bind the respective ligand with high affinity (29). The structural analysis of the complex between growth hormone and its receptor indicated that a single molecule of growth hormone bridges two receptor molecules to induce its dimerization (30). Since G-CSF and growth hormone have a similar tertiary structure (31), G-CSF also seems to induce dimerization of the receptor to activate. Here we have shown that binding of polyclonal anti-G-CSFR antibody to G-CSFR activated the G-CSF cytoplasmic region to transduce the growth signal. Furthermore, binding of FasL to a chimera consisting of the Fas extracellular and G-CSFR cytoplasmic regions also activated the chimeric receptor for the growth signal. The G-CSFR antibody is polyvalent, and FasL is a trimer (12). Activation of the receptor by these molecules suggested that not only dimeric but also the trimeric or oligomeric...
structure of the G-CSFR can transduce the signal (Fig. 6).

TNF-α and TNF-β have a trimeric structure (32–34). X-ray analysis indicated that a TNF-β trimer makes a complex with three molecules of the extracellular region of the TNF receptor (35), suggesting that TNF induces trimerization of the receptor to transduce the signal. However, it was not clear whether the bringing together of only two receptor molecules would be sufficient for signal transduction. FasL is a member of the TNF family (11) and exists as a trimer (12). Here we have shown that G-CSF, which induces dimerization of G-CSFR, could not activate the Fas cytoplasmic region in the chimera consisting of the G-CSFR extracellular and Fas cytoplasmic regions, whereas polyvalent anti-G-CSFR antibody activated the Fas cytoplasmic region of the chimera and killed the cell. These results indicated that dimerization of Fas cytoplasmic region is not sufficient to transduce the signal, and it should be oligomerized (probably, trimerized) to cause apoptosis. Previously, Dhein et al. (36) observed that the F(ab)2 fragment of the monoclonal anti-APO-1 antibody recognizing Fas did not cause apoptosis, but it induced apoptosis if they were cross-linked with the anti-F(ab)2 antibody (36). Several groups have recently isolated Fas-associated cytoplasmic proteins (FADD/MORT1, RIP) by the yeast two-hybrid system (37–39), and one of them (FADD/MORT1) was found to bind Fas after its oligomerization (40). These findings agree with the notion that an oligomer (probably trimer) but not a dimer of Fas can transduce the signal. In hematopoietic growth factor receptors that work as a dimer, the signal transducers such as JAK kinases and STAT proteins also work as dimers (hetero- or homodimer) (41, 42). In this regard, it would be interesting to examine whether the signal transducers of Fas-mediated apoptosis in the cytoplasm work also as a trimer.

We showed that the extracellular and cytoplasmic regions of the different cytokine receptor families can be functionally exchanged. There are many orphan receptors of which ligands have not been identified. The successful swapping between members of the different receptor families suggests that a chimeric receptor consisting of the extracellular region of the orphan receptor and the cytoplasmic region of the known receptor can be used to identify the respective ligand. Specifically, the G-CSFR cytoplasmic region would be a good system since several IL-3-dependent cell lines such as FDC-P1 and BAF-B03 are strictly dependent on the signal from G-CSFR for their growth (5). By exchanging the extracellular region of G-CSFR and Fas, we have generated a death factor receptor that transduces the growth signal in response to the death factor. It will be possible to generate transgenic mice carrying such chimeric receptors using the gene-targeting strategy. In these mice, the cells that should die in response to the death factor would now grow. In the reverse orientation, it may be also possible to produce transgenic mice in which specific cells responding to a certain growth factor are killed by the growth factor. Such transgenic mice may be useful to study the function of specific cells in mammalian development.

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