Three different mRNAs encoding human granulocyte colony-stimulating factor receptor

(Granulopoiesis/cytokine receptor/placenta/polymerase chain reaction/WSXWS motif)

Rikiro Fukunaga, Yoshiyuki Seto, Seiichi Mizushima, and Shigekazu Nagata

Osaka Bioscience Institute, 6-2-4 Furuedai, Suita-shi, Osaka 565, Japan

Communicated by Charles Weissmann, August 3, 1990 (received for review June 11, 1990)

ABSTRACT Three cDNAs for the human granulocyte colony-stimulating factor (G-CSF) receptor were isolated from the cDNA libraries of human U937 leukemia cells and placenta by using a murine G-CSF receptor cDNA as the probe. The human G-CSF receptor containing 813 amino acids had a marked homology (62.5%) with its murine counterpart and consisted of extracellular, transmembrane, and cytoplasmic domains. The WSXWS motif found in members of the newly identified growth factor receptor family was also present in the extracellular domain of the human G-CSF receptor. Expression of the cloned cDNA in monkey COS cells gave rise to a protein that could specifically bind G-CSF with a high affinity (Kd, 550 pM). Two other classes of the human G-CSF receptor were also identified, one of which had a deletion of the transmembrane domain and seemed to encode a secreted, soluble receptor. The third class of the G-CSF receptor contained a 27-amino acid insertion in the cytoplasmic domain and was highly expressed in placenta.

The proliferation and differentiation of granulocytes and macrophages are regulated by a family of colony-stimulating factors (CSFs). (1). Granulocyte colony-stimulating factor (G-CSF) is known to specifically act on cells that are committed to the neutrophilic granulocyte lineage (2, 3). G-CSF is a glycoprotein of Mr 20,000–25,000 that is produced by macrophages stimulated with endotoxin (2, 3), and it plays an important role in granulopoiesis during the inflammatory process. In addition to colony-stimulating activity in a semisolid culture of bone marrow cells, G-CSF has the ability to induce differentiation of some myeloid leukemia cells into granulocytes and monocytes and can also support the proliferation of other myeloid leukemia cells (2, 3).

G-CSF exerts its biological effects through interaction with specific cell-surface receptors (4). Binding studies with radiolabeled G-CSF have indicated that the G-CSF receptor is expressed not only by progenitor and mature neutrophilic granulocytes (5–7) but also by nonhemopoietic cells, such as placental cells (8), endothelial cells (9), and various carcinoma cell lines (10, 11). The number of receptors is around 300–2000 per cell, and G-CSF binds to its receptor with a Kd of 100–500 pM (5–7). We have recently purified the murine G-CSF receptor from the mouse myeloid leukemia cell line NFS-60 and shown that it has a Mr of 100,000–130,000 (7). Furthermore, recent cloning of murine G-CSF receptor cDNA from mouse NFS-60 cells has revealed that it contains 812 amino acids and consists of the extracellular, transmembrane, and cytoplasmic domains (12).

Administration of recombinant G-CSF to patients suffering from neutropenia due to various causes has suggested that G-CSF is beneficial as an adjuvant in chemotherapy and in bone marrow transplantation (13). However, to more effectively apply G-CSF in the clinical setting, it is important to examine the structure and expression of the human G-CSF receptor, since some human myeloid leukemia cells (14) or various carcinoma cells can proliferate in response to G-CSF (10, 11).

In this report, we describe the molecular cloning and characterization of human G-CSF receptor cDNAs. The human G-CSF receptor was found to be abundantly expressed in the human placenta and analysis of the cDNAs showed that there are at least two variants of the human G-CSF receptor.

MATERIALS AND METHODS

Isolation of Human G-CSF Receptor cDNA Clones. A U937 cDNA library containing cDNAs >2.5 kilobases (kb) was prepared as described (12) with the mammalian expression vector pEF-BOS (15). A total of 3.4 × 10⁶ clones was screened by colony hybridization with a 2.5-kb HindIII/Xba I DNA fragment of murine G-CSF receptor cDNA used as a probe. Hybridization was carried out as described (16), except that the hybridization temperature was lowered to 28°C, and the filter was washed at 37°C in 150 mM NaCl/15 mM sodium citrate, pH 7.0/0.1% NaDodSO₄. A human placental cDNA library prepared in λgt11 (Clontech) was screened by plaque-hybridization as described above.

Construction of Expression Plasmids, Labeled G-CSF, and Binding Assays. To construct a full-length cDNA containing the insertion in the cytoplasmic domain, plasmid pHG11 was digested completely with Nhe I and partially with BsrXI (at nucleotide position 1425). The 1.38-kb BsrXI/Nhe I fragment was then ligated with the 6.9-kb BsrXI/Nhe I fragment of pHQ3 to yield pQW11. Labeling of murine recombinant G-CSF, transfection of COS cells with mammalian expression plasmids containing human G-CSF receptor cDNA, and binding assays of I²²I-labeled G-CSF (I²²I-G-CSF) to COS cells were carried out as described (7, 12).

Analysis of RNA by Polymerase Chain Reaction (PCR). Single-stranded cDNA synthesis and the PCR were carried out as described by Kawasaki (17). In brief, 2 μg of total or poly(A) RNA was used as a template for cDNA synthesis in 50 μl of reaction mixture with 0.5 μg of random hexamer and 80 units of avian myeloblastosis virus reverse transcriptase (17). An aliquot (5 μl) of the reaction mixture was diluted with 100 μl of PCR buffer (17) containing 50 pmol each of the upstream and downstream primers and was placed in a DNA thermal cycler (Perkin–Elmer/Cetus) preheated to 80°C. The reaction was started by adding 2.5 units of Thermus aquaticus DNA polymerase (Taq polymerase), and the conditions were 94°C for 3 min, 37°C for 2 min, and 72°C for 2 min. PCR was performed for 25 cycles.

Abbreviations: CSF, colony-stimulating factor; G-CSF, granulocyte CSF; GM-CSF, granulocyte-macrophage CSF; PCR, polymerase chain reaction; IL, interleukin.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M38025, M38026, and M38027).
for the PCR were 1.5 min at 95°C, 1.5 min at 70°C, and 1.5 min at 72°C for 30 cycles.

**General Procedures.** Total RNA was prepared from various cell lines and from fresh human full-term placenta as described (18). Northern hybridization was carried out under stringent conditions (16) with the 3-kb Xho I DNA fragment of pHQ3.

For DNA sequencing analysis, the DNA fragment was subcloned in the pBluescript SK(+) vector (Stratagene), and a series of deletion plasmids was generated by using exonuclease III and mung bean nuclease (18). The sequencing reaction was performed by the dideoxynucleotide chain-termination method (18) using T7 DNA polymerase, deaza-dGTP, and deoxyadenosine 5'-[α-35S]thiophosphate.

**RESULTS**

**Isolation and Characterization of Human G-CSF Receptor cDNAs.** Binding studies using radioactive G-CSF have suggested that human U937 and placental cells express the G-CSF receptor on their surfaces (6, 8). To isolate cDNA clones containing the sequence for the human G-CSF receptor, a U937 cDNA library was screened as described in Materials and Methods, and 5 positive clones were identified (pHG1-pHG5). A human placental cDNA library (1.5 × 10^6 clones) was also screened by plaque hybridization with murine G-CSF receptor cDNA. More than 100 clones gave positive signals, and the EcoRI DNA fragments of 6 positive clones were subcloned in pBluescript SK(+) (pHG series).

As shown in Fig. 1, the restriction enzyme mapping and DNA sequencing analysis of the 9 cDNA clones revealed that they could be divided into three classes. Most of the cDNA clones (6 clones) isolated from the U937 and placental cDNA libraries belonged to the first class, which is represented by the clones pHQ3 and pHG12. The plasmids of pHQ3 and pHG12 contained a large open reading frame that encoded a protein consisting of 836 amino acids (Fig. 2). Hydropathy analysis of the predicted protein sequence indicated that the N-terminal 23 amino acid residues corresponded to the signal sequence, while the following 604, 26, and 183 residues constituted the extracellular, transmembrane, and cytoplasmic domains, respectively. The mature human G-CSF receptor thus consists of 813 amino acids with a calculated M_r of 89,743. This M_r differs by 30,000–60,000 from that reported for the native human G-CSF receptor (8), which may be explained by N-glycosylation on some of the nine potential N-glycosylation sites in the extracellular domain of the receptor. The overall similarity of the human G-CSF receptor to the murine G-CSF receptor was 72% at the nucleotide sequence level and 62.5% at the amino acid sequence level. The WsxWx motif that is conserved in the members of the cytokine receptor family (19–21) can be found in the extracellular domain.

The second class of cDNA is represented by the clone pHQ2, which was derived from the U937 cDNA library. The nucleotide sequence of pHQ2 was identical to that of pHQ3, except that it lacked 88 nucleotides from positions 2034 to 2121, including the region encoding the transmembrane domain (Figs. 1 and 2). This deletion resulted in an altered translation reading frame that encoded an additional 150 amino acids after the deletion point. Thus, the polypeptide encoded by pHQ2 seems to be a secreted, soluble form of the G-CSF receptor, consisting of 748 amino acids with a calculated M_r of 82,707.

As shown in Figs. 1 and 2, two clones (pHG11 and pHG5) isolated from the placental cDNA library were distinguished from other clones by having an 81-base-pair (bp) insertion at nucleotide 2210, and these represent the third class of the G-CSF receptor. The insertion was in the cytoplasmic domain of the G-CSF receptor and it did not change the translational reading frame. The putative polypeptide encoded by this class of cDNA, therefore, is 27 amino acids larger (M_r 2957) than that encoded by the class I G-CSF receptor cDNA.

**Binding Characteristics of the Cloned Human G-CSF Receptors.** We examined the binding of 125I-G-CSF to the recombinant human G-CSF receptor expressed in COS cells. As shown in Fig. 3, murine 125I-G-CSF bound to COS cells transfected with pHQ3 in a saturating manner. Scatchard analysis of the specific binding revealed a single species of binding site, with an equilibrium dissociation constant of 550 pM and 3.4 × 10^5 receptors per cell. The dissociation constant for the binding of murine G-CSF to human G-CSF receptors expressed in COS cells was similar to that observed for the binding of murine G-CSF to murine G-CSF receptors expressed in COS cells (Fig. 3). Since the native human G-CSF receptor expressed in U937 cells can bind human G-CSF with an equilibrium dissociation constant of 420 pM (6), these results suggested that the polypeptide encoded by the cDNA in pHQ3 is sufficient to express the high-affinity receptor for human G-CSF.

To examine the binding properties of the third class of G-CSF receptors, a full-length cDNA was constructed by using the 5' half of the pHQ3 cDNA and the 3' half of the pHG11 cDNA, and it was inserted into the mammalian expression vector pEF-BOS (designated as pQW11). COS cells transfected with pQW11 could bind labeled G-CSF with a K_d similar to that observed for COS cells transfected with pHQ3 (Fig. 3), indicating that the 27-amino acid insertion in the cytoplasmic domain of the receptor has little effect on the binding of G-CSF to the receptor. When COS cells were transfected with the second class of cDNA (pHQ2), a low level of binding of 125I-G-CSF was observed (Fig. 3A). As shown in Fig. 3B, Scatchard analysis of the specific binding revealed that there were 6 × 10^5 binding sites per cell with a K_d of 440 pM. These results suggested that the receptor encoded by pHQ2, which lacks the transmembrane domain, was probably secreted by the cells. However, we do not understand why a low but significant amount of the membrane-bound receptor was found in COS cells transfected with pHQ2.

**Analysis of G-CSF Receptor Transcript.** RNA from various human cell lines and from placental tissue was analyzed by Northern hybridization using radiolabeled human G-CSF

![Fig. 1. Diagram and restriction map of the three human G-CSF receptor cDNAs. Boxes represent the open reading frames, of which the shaded and solid regions, respectively, indicate the signal sequence and the transmembrane domain for the G-CSF receptor. The hatched region in pHQ2 indicates where the amino acid sequence differs from those in the other clones as a result of an altered open reading frame. The hatched box in pHG11 shows the inserted sequence of 27 amino acids.](image-url)
receptor cDNA. As shown in Fig. 4A, a single band of 3.7 kb was observed in the RNAs from U937, KG-1, and placenta, but not in the RNAs from HL-60, FL, or CHU-2 cells. Unexpectedly, the signal detected in RNA from the placenta was >20 times stronger than that detected with RNA from either U937 or KG-1 cells. When the blot was rehybridized with 32P-labeled human elongation factor 1α cDNA (22), the signals were almost similar (Fig. 4B). These results indicate that the placenta expresses G-CSF receptor mRNA more abundantly than myeloid leukemia cells.

As described above, we isolated three different human G-CSF receptor cDNAs from the U937 and placental cDNA libraries (Figs. 1 and 2). To examine which mRNA is expressed by these cells, two sets of forward and reverse primers were synthesized, and the specific cDNAs were amplified by the PCR method. Using the first set of oligonucleotides (nucleotides from positions 1790–1810 for the forward primer and 2179–2156 for the reverse primer), amplification of RNA from U937 or placental cells gave a major band (A1) of 390 bp, which corresponded to the class I human G-CSF receptor as represented by pHQ3 or pHG12. In addition, U937 mRNA gave a faint band (A2) of 302 bp, which seemed to correspond to the cDNA containing the deletion in the transmembrane domain (Fig. 5, lanes 1–3). On the other hand, when the second set of oligonucleotides (nucleotides from positions 2086–2105 and 2322–2303) was used as the specific primers, placental cDNA gave two bands of 318 bp (B1) and 237 bp (B2), which appeared to be derived from the class III and class I mRNAs, respectively (lanes 4–6). These assumptions were confirmed by the sequencing of the DNA fragments (A1, A2, B1, and B2) after subcloning in M13mp9 vector. Several other high molecular weight bands seen in Fig. 5 were not characterized. They may correspond to the cDNAs for unspliced RNAs or other variants of the receptor. From these results, it can be concluded that both U937 and placental cells express the class I G-CSF receptor. In addition, U937 cells express the soluble form of the G-CSF receptor, while the G-CSF receptor containing the insertion in the cytoplasmic domain is expressed at a significant level by placental cells.

**DISCUSSION**

In this study, we isolated cDNAs for the human G-CSF receptor by cross-hybridization with murine G-CSF receptor cDNA. The amino acid sequence of the human G-CSF receptor (Fig. 2) was highly homologous to that of the murine G-CSF receptor, in agreement with the observation that murine G-CSF could bind to the recombinant human and murine G-CSF receptors with almost identical dissociation constants (Fig. 3). In the murine G-CSF receptor, there are regions that have similarities to some members of the cytokine receptor family, chicken contactin, and the interleukin 4 (IL-4) receptor (12). The amino acid sequences of these regions are also highly conserved in the human G-CSF receptor and confirm that the G-CSF receptor is a member of the newly identified cytokine receptor family (19-21).
human primary leukemia cells by Northern hybridization and the PCR method and to study the functions of the soluble receptor.

This study identified at least three different mRNAs for the human G-CSF receptor (Figs. 1 and 2), which had nucleotide sequences that were identical except for specific deletions or insertions. Since the human genome seems to contain a single gene for the human G-CSF receptor (unpublished results), these different mRNAs are probably produced by alternative splicings from a single precursor RNA. Characterization of the human chromosomal gene for the G-CSF receptor will allow clarification of the mechanism that generates different mRNAs for the G-CSF receptor.

We thank Dr. S. Tsubokura for providing fresh human placental tissue. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.