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Identification and characterization of receptors for granulocyte colony-stimulating factor on human placenta and trophoblastic cells

(mutein/radioiodination/cross-linking/receptor structure)

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ABSTRACT **Since radioiodination of human granulocyte colony-stimulating factor (G-CSF) is difficult, we synthesized a mutein of human G-CSF that retains full biological activity and receptor-binding capacity for at least 2 weeks after radioiodination. Receptors for human G-CSF were characterized in the plasma membrane fraction from the human term placenta (human placental membranes) and trophoblastic cells by using the ^{125}I-labeled mutein of human G-CSF (KW-2228). The specific binding of ^{125}I-labeled KW-2228 to placental membranes was pH-dependent, with maximal specific binding at pH 7.8; it increased linearly with protein to 3.7 mg of protein per ml and was both time- and temperature-dependent, with maximal binding at 4°C after a 24-hr incubation. When we examined the ability of hematopoietic growth factors to inhibit ^{125}I-labeled KW-2228 binding, we found that KW-2228 and intact human G-CSF inhibited ^{125}I-labeled KW-2228 binding, whereas erythropoietin or granulocyte–macrophage colony-stimulating factor did not. Scatchard analysis revealed a single receptor type with a B_{max} of 210 fmol/mg of protein and a K_d of 480 pM. The human G-CSF receptors on human placental membranes were shown to consist of two molecular species of 150 kDa and 120 kDa that could be specifically cross-linked to ^{125}I-labeled KW-2228. Human trophoblastic cells, T3M-3, also possessed a single receptor for G-CSF with a B_{max} of 533 receptors per cell and a K_d of 390 pM. Thus we have identified the receptor for human G-CSF on human placental membranes and trophoblastic cells, and the presence of this receptor in these membranes suggests that human G-CSF plays some role in the feto–placental unit during human development.**

Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein growth factor required for the proliferation and differentiation of progenitors of neutrophils (1). G-CSF is synthesized by macrophages (2) and placental tissues (3, 4).

Studies on G-CSF receptors have been hampered because radioiodination of human G-CSF causes a significant decrease in its biological activity and receptor-binding capacity, and thus the distribution of G-CSF receptors in human tissues has not been fully elucidated. To investigate the mechanism of G-CSF action on its target cells, we developed a mutein of human G-CSF that retains full biological activity and receptor-binding capacity even 2 weeks after radioiodination. By using the radiolabeled mutein G-CSF, receptors for human G-CSF were identified on human neutrophils (5). Interestingly, specific binding of human G-CSF was observed on human placental membranes.

In this report, we describe the identification of receptors for human G-CSF in placental tissues and trophoblastic cells.

MATERIALS AND METHODS

For the binding experiments, Na^{125}I (Amersham), Enzymobead reagent (Bio-Rad), and disuccinimidyl suberate (Pierce) were used. Culture media and fetal bovine serum were obtained from Flow Laboratories. Aprotinin, phenylmethylsulfonyl fluoride, dithiothreitol, EDTA, β-D-glucose, bovine serum albumin, NaN_3, and Hepes were purchased from Sigma. KW-2228 and intact *Escherichia coli*-derived G-CSF were kindly provided by Kyowa Hakko, Kogyo, Japan. Erythropoietin (Toyobo, Tokyo) and granulocyte–macrophage colony-stimulating factor (Genzyme) were also purchased.

Preparation of Muteins of Human G-CSF. Clones of cDNA encoding human G-CSF were isolated from human cancer cells (6, 7) and from circulating human monocytes as described (2). A large quantity of recombinant human G-CSF is now available (8, 9). However, intact G-CSF rapidly loses the biological activity and receptor-binding capacity a few hours after radioiodination. To obtain muteins of human G-CSF with more stable and potent biological activity, we synthesized about 100 muteins by site-directed mutagenesis, cassette mutagenesis, insertions, or deletions (10). Among these muteins, KW-2228, in which Thr-1, Leu-3, Gly-4, Pro-5, and Cys-7 (2, 6, 7) were replaced with Ala, Thr, Tyr, Arg, and Ser, respectively, showed more potent G-CSF activity than intact human G-CSF *in vitro* and *in vivo* (10). Biological activity and receptor-binding capacity were retained for at least 2 weeks after radioiodination.

Preparation of Radioiodinated KW-2228. *E. coli*-derived mutein G-CSF (KW-2228, more than 99% pure) was radioiodinated with 0.5 mCi of Na^{125}I (1 Ci = 37 GBq) by using solid-phase glucose oxidase–lactoperoxidase (11). The radioiodination was performed as described (5). The specific activity of radioiodinated KW-2228 was 10^7 cpm/μg of protein.

Preparation of Human Placental Membranes. A crude membrane fraction containing microsomal and plasma membranes (100,000 × *g* pellet) was prepared from normal human fresh-frozen full-term placenta, as described by Posner (12). Homogenizing buffer contained aprotinin (200 units/ml or 1 trypsin inhibitor unit/ml) and 1 mM phenylmethylsulfonyl fluoride.

Cell Culture. A human trophoblastic cell line, T3M-3, was established as described (13). T3M-3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. Subcultures were prepared by using 0.25% trypsin and 0.02% EDTA as described (13).

Binding Experiments. Unless otherwise stated, the binding experiments using human placental membranes were per-

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Abbreviation: G-CSF, granulocyte colony-stimulating factor.
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formed for 24 hr at 4°C in a total volume of 500 μl of 50 mM Hepes buffer (pH 7.8) containing 5 mM $MgSO_4$, 0.1% bovine serum albumin, 50,000 cpm of ^{125}I-labeled KW-2228 (5 ng), and 850 μg of placental membrane protein with or without KW-2228 (200 ng/ml) in 1.5-ml Microfuge tubes (Bioplastics, Osaka, Japan). After incubation, Microfuge tubes were centrifuged in a Beckman Microfuge B for 1 min and the supernatant was aspirated. The pellet was washed with 50 mM Hepes buffer (pH 7.8) and centrifuged again in a Beckman Microfuge B. The radioactivity of the resultant pellet was measured in an Aloka auto well gamma system (ARC-2511).

The binding experiments using choriocarcinoma cells (3–5 × 10^6 cells) were performed at 4°C for 1 hr in 12-well tissue culture plates (Costar) in 1 ml of culture medium containing 50,000 cpm of ^{125}I-labeled KW-2228 with or without KW-2228 (5 μg/ml). After incubation, the medium was aspirated and the cells were washed twice with cold isotonic phosphate-buffered (0.01 M phosphate, pH 7.4) saline. The cells were then solubilized in 1 ml of 1 M NaOH, and the radioactivity was measured.

The specific binding was determined as total binding minus binding in the presence of excess unlabeled KW-2228.

Chemical Cross-Linking. The membrane pellet was resuspended in 300 μl of 50 mM Hepes (pH 7.8) containing various concentrations of disuccinimidyl suberate in 1.5-ml Microfuge tubes and incubated at 4°C for 30 min. The reaction was quenched by the addition of 900 μl of 10 mM Tris·HCl (pH 7.4) containing 1 mM EDTA. The resultant pellet was solubilized in 2× Laemmli sample buffer (14) in the presence or absence of 200 mM dithiothreitol. The sample was then boiled and stored at −70°C until use.

Electrophoresis and Autoradiography. Electrophoresis was performed by the method of Laemmli (14). The sample was put onto 8% polyacrylamide/$NaDodSO_4$ gels. The gels were stained [50% (wt/vol) trichloroacetic acid/0.25% Coomassie blue], destained [7% (vol/vol) acetic acid], and autoradiographed using Kodak X-Omat OAR film.

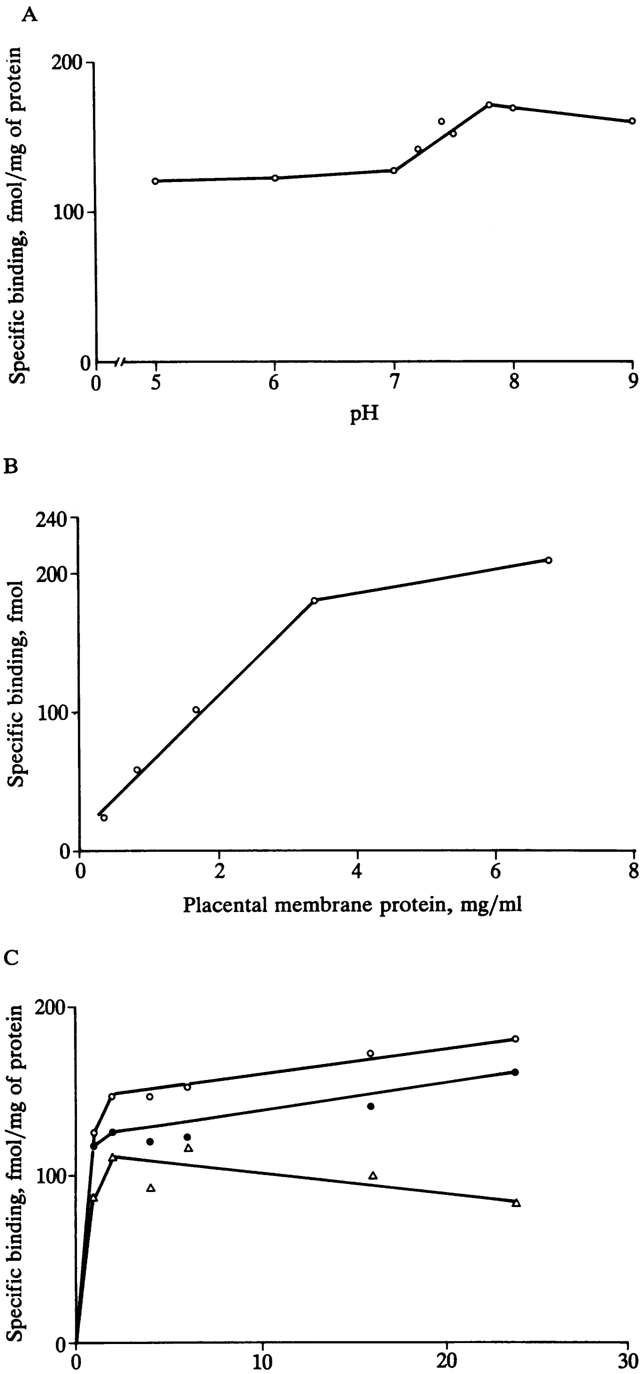

FIG. 1. Specific binding of ^{125}I-labeled KW-2228. (*A*) Effect of pH on the specific binding of ^{125}I-labeled KW-2228 to placental membranes. (*B*) Specific binding of ^{125}I-labeled KW-2228 as a function of protein concentration. (*C*) Time and temperature dependency of the specific binding of ^{125}I-labeled KW-2228 in placental membranes. Incubation was performed at 4°C (○), 22°C (●), and 37°C (△).

RESULTS

The specific binding of ^{125}I-labeled KW-2228 to human placental membranes was pH-dependent and reached maximum at pH 7.8 (Fig. 1*A*). Fig. 1*B* shows the dependency of the specific binding of ^{125}I-labeled KW-2228 on the protein concentration of human placental membranes. The increase in the specific binding was directly related to the protein concentration up to 3.4 mg of protein per ml. The specific binding was time- and temperature-dependent (Fig. 1*C*). Higher specific binding was observed at 4°C than at 22°C or 37°C. Maximum specific binding was observed at 4°C with a 24-hr incubation period (approximately 180 fmol/mg of protein). The concentration-dependent inhibition of ^{125}I-labeled KW-2228 binding by hematopoietic growth factors is shown in Fig. 2. Both KW-2228 and intact *E.coli*-derived G-CSF

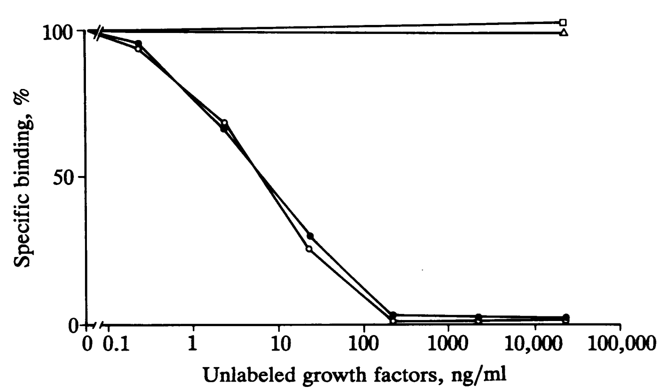

FIG. 2. Inhibition of specific ^{125}I-labeled KW-2228 binding to placental membranes by unlabeled KW-2228 or intact *E. coli*-derived human G-CSF. ○, KW-2228; ●, intact *E. coli*-derived G-CSF; △, granulocyte–macrophage colony-stimulating factor; □, erythropoietin. Nonspecific binding (binding in the presence of G-CSF at 200 ng/ml) was subtracted from total binding to determine specific binding and was 30–40% of total binding. Specifically bound ^{125}I-labeled KW-2228 was expressed as a percentage of the specific binding measured in the absence of added growth factor.

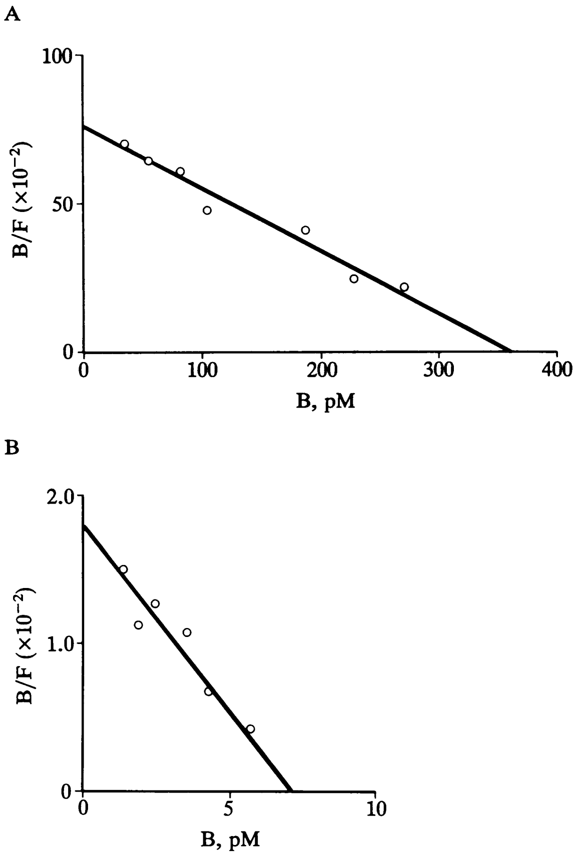

FIG. 3. (*A*) Scatchard analysis of the specific binding data of [125]I-labeled KW-2228 to placental membranes. (*B*) Scatchard analysis of the specific binding of [125]I-labeled KW-2228 to T3M-3 cells (4×10^6 cells). Nonspecific binding (binding in the presence of KW-2228 at 5 μg/ml) was subtracted from total binding to determine specific binding and was 40–50% of total binding.

inhibited [125]I-labeled KW-2228 binding in a concentration-dependent manner with the same IC_{50} value (5 ng/ml). KW-2228 and *E. coli*-derived G-CSF (200 ng/ml) completely inhibited the binding of [125]I-labeled KW-2228. Erythropoietin and granulocyte–macrophage colony-stimulating factor (up to 20 μg/ml) did not inhibit [125]I-labeled KW-2228 binding.

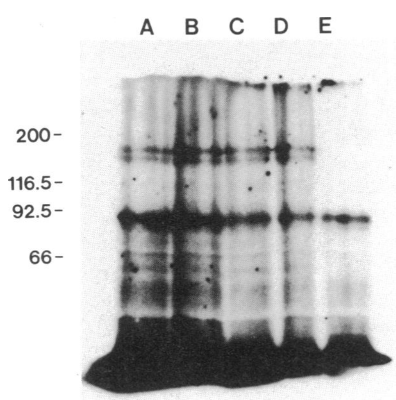

FIG. 4. Inhibition of the formation of a cross-linked complex by unlabeled KW-2228 as shown by autoradiography. The pelleted complex was incubated with disuccinimidyl suberate for 30 min at 4°C to give the following final disuccinimidyl suberate concentrations. Lanes: A, 0.01 mM; B, 0.1 mM; C, 0.25 mM; D, 0.5 mM; E, 0.5 mM. Unlabeled KW-2228 was added as follows. Lanes A, B, C, and D, unlabeled KW-2228 was not added; E, unlabeled KW-2228 at 2 μg/ml was added. Molecular masses in kDa are shown to the left.

Scatchard analysis (15) of the specific binding of [125]I-labeled KW-2228 to human placental membranes is shown in Fig. 3*A*. The results demonstrate that a single type of G-CSF receptor exists in human placental membranes. The B_{max} calculated from the intercept of the slope of the Scatchard plot on the abscissa was 210 fmol/mg of protein. The apparent dissociation constant (K_d) was 480 pM. Representative results of the chemical cross-linking experiments are shown in Fig. 4. Radioactive bands showing apparent molecular masses of 170 kDa and 140 kDa appeared under reducing conditions. These two radioactive bands disappeared in the presence of KW-2228 at 2 μg/ml (Fig. 4). The same results were obtained under nonreducing conditions (data not shown).

Specific binding of [125]I-labeled KW-2228 was observed to trophoblastic cells. The number of receptors per T3M-3 trophoblastic cell, calculated from the intercept of the abscissa in Fig. 3*B*, was 533. The apparent dissociation constant (K_d) was 390 pM.

DISCUSSION

We have demonstrated the existence of a single receptor type for human G-CSF on placental membranes and trophoblastic cells. Recently, placenta and trophoblastic cells have been shown to express c-fms mRNA and c-*fms* protooncogene product (M-CSF receptor), respectively (16, 17). Pollard *et al.* (18) have reported that M-CSF regulates placental trophoblast proliferation and differentiation. Our results on placental G-CSF receptors plus the reports (3, 4) that G-CSF is synthesized by placental tissue suggest that this lineage-specific hematopoietic growth factor also has an important role on the development of the feto–placental unit.

Another interesting finding of this paper is that the structure of G-CSF receptor in placental membranes differs from that in hematopoietic cells or cell lines. Scatchard analysis of the data gives a linear slope (Fig. 3*A*), indicating that G-CSF binds to a single type of binding site in placental membranes and the trophoblastic cells. Although some variations are marked, all of the cells possessed a single receptor type for human G-CSF. However, chemical cross-linking data showed two radioactive bands that disappeared in the presence of unlabeled KW-2228 under reducing conditions (Fig. 4). These two cross-linked complexes (170 kDa and 140 kDa) were not disulfide-linked subunits of a single receptor complex since their apparent molecular masses were the same under reducing or nonreducing conditions. In contrast, only one cross-linked 170-kDa complex was identified in human neutrophils (5) and murine cell lines (19). After subtraction of the molecular mass of KW-2228 (approximately 20 kDa), the two binding species had apparent molecular masses of 150 kDa and 120 kDa. Since the data from the Scatchard analysis showed only one type of affinity for the receptor, these two bands do not appear to represent two classes of binding sites, such as low- and high-affinity receptors. These results suggest that the two bands may represent two distinct receptors for [125]I-labeled KW-2228 with approximately equal affinity. It is also possible that 120-kDa protein is a less glycosylated form of the 150-kDa receptor or proteolytic fragment of 150-kDa receptor. Structural variations of receptors for peptide growth factors have been reported. The α subunits of insulin receptors and insulin-like growth factor I receptors in central nervous system tissues were shown to have smaller molecular masses than the receptors found in peripheral target tissues (20, 21). These variations can be attributed to the difference in carbohydrate moiety of the receptor molecules. Whether the structural difference of G-CSF receptors between placental tissues and hematopoietic cells represents the functional diversity of this lineage-specific hematopoietic growth factor will have to be ascertained by further studies.

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