Journal of Cellular Biochemistry 103:1512–1523 (2008)

The Granulocyte Colony Stimulating Factor (G-CSF) Activates Jak/STAT and MAPK Pathways in a Trophoblastic Cell Line

Verónica Julieta Marino and Leonor Patricia Roguin*

Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Buenos Aires, Argentina

Abstract Granulocyte colony-stimulating factor receptor (G-CSFR) has been found in placenta tissues, although its functional role has not yet been defined. In order to explore the molecular pathways induced by G-CSF in this tissue, we first reveal the presence of G-CSFR in the JEG-3 human trophoblastic cell line and then examined the phosphorylation of Janus tyrosine kinases (Jak), signal transducers and activators of transcription (STAT) proteins and mitogen-activated protein kinases (MAPK) after G-CSF binding to receptors. We showed that Jak1, Jak2, Tyk2, and STAT3 were phosphorylated after incubation with G-CSF. Phosphorylation of p38 and p44/42 MAPK was also activated by G-CSF, and specifically blocked in the presence of the corresponding inhibitors. Similar intracellular pathways were induced by G-CSF in a myeloid leukemia NFS-60 cell line that was studied in parallel. Conversely to cytokine action in myeloid cells, G-CSF did not induce a proliferative response in JEG-3 cells. When the effect of G-CSF on cellular viability was evaluated, cytokine-stimulated JEG-3 cells were protected from foetal serum starvation. In addition, when JEG-3 cells deprived of serum were incubated at different times in the presence of G-CSF, a progressive decrease in the percentage of hypodiploid cells was observed. In summary, we identified the molecular pathways activated after G-CSF binding to trophoblastic cell receptors and showed that G-CSF behaved as a protective cytokine, which supports JEG-3 cells survival. J. Cell. Biochem. 103: 1512–1523, 2008.

Key words: granulocyte colony-stimulating factor; trophoblastic cells; signal transduction; cell survival

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF), a member of the cytokine family of growth factors, stimulates the proliferation, differentiation, survival, and functional activation of cells of the granulocyte lineage [Morstyng and Burgess, 1988; Nicola, 1989; Demetri and Griffin, 1991]. G-CSF exerts its biological activities through its interaction with a specific receptor expressed on the surface of target cells, the G-CSFR, which is a member of the hematopoietic receptor superfamily [Morstyng and Burgess, 1988; Nicola, 1989; Demetri and Griffin, 1991]. The presence of G-CSFR has been reported in normal myeloid progenitors, mature neutrophils and some myeloid and lymphocytic leukemia cell lines [Nicola and Metcalf, 1985; Begley et al., 1988; Shimoda et al., 1992; Shinjo et al., 1995; Handa et al., 2000]. In addition, G-CSFR has also been detected in non-hematopoietic tissues, including endothelial cells [Bussolino et al., 1989], cardiomyocytes and cardiac fibroblasts [Harada et al., 2005], neural stem cells [Jung et al., 2006], trophoblastic cells and human placenta [Uzumaki et al., 1989; Shorter et al., 1992; Mc Cracken et al., 1996]. Particularly, the synthesis of G-CSF and the expression of its receptor have been previously studied in human placenta. Thus, it has been reported that stromal cells from both foetal chorionic villi and maternal decidual tissues express G-CSF throughout pregnancy [Shorter et al., 1992; Mc Cracken

Grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica; Grant number: PICT11161; Grant sponsor: Universidad de Buenos Aires; Grant number: B009; Grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas; Grant number: PIP 5553.

*Correspondence to: Leonor Patricia Roguin, Instituto de Química y Fisicoquímica Biológicas (IQUIFIB), Facultad de Farmacia y Bioquímica, Junín 956, C1113AAD Buenos Aires, Argentina. E-mail: rvroguin@qb.ffyb.uba.ar

Received 18 April 2007; Accepted 17 July 2007

DOI 10.1002/jcb.21542.

et al., 1996], whereas G-CSFR is mainly detected in first and third trimester placental tissues [Mc Cracken et al., 1996, 1999].

Signal transduction pathways activated after G-CSF binding to hematopoietic receptors are connected to the receptor-associated Janus tyrosine kinases (Jak), the signal transducers and activators of transcription (STAT) proteins and the mitogen-activated protein kinases (MAPK) [Bashey et al., 1994; Nicholson et al., 1995; Avalos, 1996; Rausch and Marshall, 1999; Akbarzadeh and Layton, 2001; Srinivasa and Doshi, 2002]. Some of the signal molecules induced by G-CSF in non-hematopoietic cells, such as endothelial cells [Fuste et al., 2004], cardiomyocytes [Harada et al., 2005] and neural stem cells [Jung et al., 2006], have been recently described. However, the intracellular pathway triggered by G-CSF in the placenta tissue, a putative target for G-CSF action, remains unknown.

In the present study, in order to explore the potential action of G-CSF in trophoblastic cells, we employed human choriocarcinoma JEG-3 cells as a model system. This cell line maintains not only several of the biochemical characteristics of the normal trophoblastic cells, such as the production of human chorionic gonadotropin, human placental lactogen, progesterone and estrogen, but it has also been widely used to study trophoblast invasion [Kohler et al., 1971; Bahn et al., 1981; Mandl et al., 2002; Karmakar et al., 2004]. We first revealed the presence of G-CSFR in these cells and then examined the G-CSF-induced signaling. The effect of G-CSF on cell viability and proliferation was also studied.

MATERIALS AND METHODS

Experimental Design

Choriocarcinoma cells (JEG-3) were incubated with increasing concentrations of G-CSF or with 1 μg/ml of G-CSF for different times. The presence of G-CSF receptors was determined by using [^{125}I]G-CSF or by immunocytochemistry with anti-human G-CSF receptor antibody. G-CSF-induced Jak/STAT signaling pathways were assessed by immunoprecipitation, whereas phosphorylation of p38 and p44/42 MAPK was determined by Western blot assays. JEG-3 cell viability was studied after the addition of G-CSF to the culture medium and the amount of hypodiploid cells was evaluated by flow cytometry.

Cytokines

Recombinant human G-CSF expressed in Chinese hamster ovary cells was supplied by BIO SIDUS S.A., Buenos Aires, Argentina. Murine recombinant Interleukin 3 (IL-3) was purchased from Gibco BRL, Gaithersburg, MD. Radioiodination of the recombinant G-CSF was performed using lactoperoxidase as previously described [Marino et al., 2001]; specific activity ranged from 60 to 80 μCi/μg. Recombinant interferon-α2b (IFN-α2b) and erythropoietin (EPO) were provided by BIO SIDUS S.A Interleukin-6 (IL-6) was obtained from Gibco BRL, human growth hormone (hGH) and human placental lactogen (hPL) were supplied by the NIDDK's National Hormone and Pituitary Program and A.F. Parlow, Harbor-UCLA Medical Center, Torrance, CA.

Cell Cultures

Choriocarcinoma cells (JEG-3) were maintained in Minimum Essential Medium (MEM, Gibco BRL) containing 15% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. NFS-60 cell line, derived from murine myeloid leukemia, is responsive to different growth factors including G-CSF or IL-3 [Hara et al., 1988]. Cells were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 5% FBS, 4 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.4 ng/ml of mouse IL-3.

Detection of G-CSF Receptors by Immunocytochemistry

Human choriocarcinoma JEG-3 cells were plated on coverslips, grown up to 70% confluence and fixed 5 min at room temperature with 4% paraformaldehyde. After washing with PBS, monolayers were blocked with PBS/BSA 1% and incubated 2 h with 10 μg/ml of either monoclonal antibody anti-G-CSF receptor or normal mouse IgG as negative control (R&D Systems, Inc., Minneapolis, MN). Then, coverslips were rinsed, incubated 1 h with a dilution 1:100 of goat anti-mouse fluorescein (FITC) conjugated IgG (Becton Dickinson, San José, CA) and visualized with an Olympus BX50 epifluorescence microscope provided with a Cool-Snap digital camera.

Binding of [^{125}I]G-CSF to JEG-3 Cell Receptors

JEG-3 cells (2×10^5 cells/well) were seeded into 35 mm 6-well tissue culture plates in 3 ml of MEM containing 1% FBS and allowed to grow 72 h until the cultures reached confluence ($\sim 7 \times 10^5$ cells/well). Monolayers were washed twice with cold PBS and then incubated for 4 h at 4°C with 300,000 c.p.m. of [^{125}I]G-CSF and increasing concentrations of unlabeled G-CSF or other cytokines in a total volume of 1 ml of MEM, 1% FBS. Binding reaction was finished by aspirating the tissue culture medium and washing the wells carefully twice with cold PBS. Cells were detached and resuspended in 0.2 ml of 0.25% trypsin, 0.03% EDTA. Bound radioactivity was counted in an automatic gamma counter. Non-specific binding was measured in the presence of 1 μg/ml of unlabeled G-CSF. Binding affinity constant and capacity values were calculated from displacement curves by Scatchard [1949] analysis.

Immunoprecipitation of Jak/STAT Proteins

Monolayers of JEG-3 cells were washed and maintained 16 h in MEM without FBS. NFS-60 cells were washed twice with PBS and incubated overnight at 37°C with RPMI 1640 medium without IL-3 for factor starvation. To measure the phosphorylation of Jaks and STAT proteins, JEG-3 cells (20×10^6) were treated with or without 1 μg/ml of G-CSF for 15 min and after aspirating the incubation medium, cells were detached with 0.25% trypsin, 0.03% EDTA. NFS-60 cells (30×10^6) were stimulated with 0.1 μg/ml of human G-CSF for 15 min and then harvested by centrifugation.

After washing with cold PBS, the cells were lysed for 30 min at 4°C in 1 ml of lysis buffer (0.5% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml trysin inhibitor, 1 μg/ml leupeptin, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 150 mM NaCl, 50 mM Tris, pH 7.4). Lysates were cleared by centrifugation for 10 min at 12,000 rpm, and the supernatants were incubated in the presence of rabbit polyclonal anti-Jak1, anti-Jak2, anti-Tyk2, anti-STAT1, or anti-STAT3 antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) overnight at 4°C with constant rocking. After incubation, 50 μl of protein A-Sepharose (50% v/v; Sigma, St. Louis, MO) were added and the preparation was further incubated 2 h at 4°C and washed twice with lysis buffer. Proteins were dissociated with sample buffer and submitted to 8% SDS–PAGE.

p38 and p44/42 MAPK Western Blots

After immunoprecipitation and 8% SDS–PAGE, proteins were transferred onto PVDF membranes (Amersham Biosciences, Piscataway, NY) for 2 h at 100 V in 25 mM Tris, 195 mM glycine, 20% methanol, pH 8.2. Non-specific antibody binding sites were blocked by incubating membranes for 1 h at room temperature with 10 mM Tris, 130 mM NaCl and 0.05% Tween-20, pH 7.4, (TBS-T), containing 3% BSA. Membranes were then incubated overnight at 4°C with anti-phosphotyrosine mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.) or the indicated antibodies diluted in TBS-T, containing 1% BSA. After 3–4 washes with TBS-T, membranes were incubated for 1 h at room temperature with anti-mouse IgG (horseradish peroxidase-conjugated goat IgG from Jackson ImmunoResearch Labs, West Grove, PA) or anti-rabbit IgG (horseradish peroxidase-conjugated goat IgG from Santa Cruz Biotechnology, Inc.) diluted in TBS-T, 1% BSA. The immunoreactive proteins were visualized using the ECL detection system (Amersham Biosciences) according to the manufacturer's instructions. For quantification of band intensity, the western blots were scanned using a densitometer (Gel Pro Analyzer).

MAPK phosphorylation was determined after western blotting of cell lysates. Briefly, JEG-3 and NFS-60 cells (1×10^6 cells) were deprived for 48 h of serum or cytokine, respectively, and then stimulated with or without G-CSF (1 μg/ml) for different times. After washing with PBS, cells were incubated with 10 μl cold lysis buffer at 4°C for 30 min. Lysates were then cleared by centrifugation for 10 min at 12,000 rpm, and the supernatants were diluted with sample buffer, submitted to 12% SDS–PAGE, and transferred onto PVDF membranes (Amersham Biosciences) as described before. Membranes were then incubated overnight at 4°C with the corresponding anti-phospho-p38 (Thr180/Tyr182) or anti-phospho-p44/42 (Thr202/Tyr204) antibodies or the specific anti-p38 or p44/42 proteins (Cell Signaling Technology, Beverly, MA). After incubation with the secondary antibody (Santa Cruz Biotechnology, Inc.), immunoreactive proteins were visualized using the ECL detection system (Amersham Biosciences)

and densitometrically analyzed. In some experiments, JEG-3 cells were pretreated for 30 min with 10 μM SB203580, a p38 MAPK inhibitor Santa Cruz Biotechnology, Inc. or 1 μM PD98059, a selective inhibitor of p44/42 MAPK kinase I (Promega Corp., Madison, WI).

Cell Viability Assays

JEG-3 cells growing in complete culture medium were tripsinized, washed twice with PBS and resuspended at 2.5×10^5 cells/ml in medium containing 10% or 0.5% FBS. A volume of 50 μl/ml of cell suspension was incubated in 96-well microplates with the same volume of medium with or without 2 μg/ml G-CSF. After incubating 72 h at 37°C, the number of viable cells was evaluated by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) assay [Cory et al., 1991] using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega Corp.).

Flow Cytometry Analysis of DNA Content

Approximately 1×10^6 JEG-3 cells were washed with PBS, resuspended in MEM in the absence of serum with or without 1 μg/ml G-CSF and then incubated for 24 or 48 h at 37°C. After harvesting and washing with PBS, 1 ml of 70% ethanol was added while vortexing and cells were kept at 4°C. To estimate the relative DNA content, cells were washed twice with PBS and resuspended in 500 μl of 0.1% sodium citrate buffer, pH 8.4, Triton X-100 0.1% and propidium iodide (50 μg/ml) overnight at 4°C. The stained cells were analyzed by a FACScan flow cytometer (Becton Dickinson).

RESULTS

Detection of G-CSF Receptors on JEG-3 Cells

The presence of G-CSF receptors on JEG-3 cells was determined by immunocytochemistry. Microscopy evaluation of monolayers showed that JEG-3 cells were clearly stained after using an specific anti-human G-CSF receptor antibody, whereas no significant fluorescence was observed when cells were incubated in the presence of normal mouse IgG used as control (Fig. 1A). In order to characterize G-CSF:receptor interaction, the cytokine was labeled with [^{125}I] and the tracer was then incubated for 4 h at 4°C with JEG-3 monolayers in the presence of increasing concentrations of unlabeled G-CSF. As shown in Figure 1B, unlabeled G-CSF inhibited [^{125}I]G-CSF binding in a dose-dependent manner. Scatchard analysis of the competitive binding data revealed a single species of binding sites with a binding affinity of 3×10^9 M^{-1} and a capacity value of 1,300 sites/cell (Fig. 1B, inset). When other cytokines, including IFN-α2b, EPO, IL-6, hGH and hPL, were incubated with [^{125}I]G-CSF, no inhibition of the cytokine binding to JEG-3 cells was observed, suggesting the specificity of the receptor (data not shown).

G-CSF Stimulates the Jak/STAT Pathway in JEG-3 Cells

Tyrosine kinases Jak1, Jak2, and Tyk2 have been reported to contribute to G-CSF-induced myeloid proliferation [Nicholson et al., 1994, 1995; Avalos, 1996; Tian et al., 1996; Shimoda et al., 1997; Akbarzadeh and Layton, 2001]. In order to establish whether G-CSF activates the Janus family kinases in the human choriocarcinoma JEG-3 cell line, tyrosine phosphorylation of Jak1, Jak2, and Tyk2 was examined both in these cells and in a G-CSF dependent myeloid cell line (NFS-60 cells). Cell lysates were submitted to immunoprecipitation with the corresponding anti-kinase antibodies and then immunoblotted with anti-phosphotyrosine antibody (anti-PY). As shown in Figure 2, a significant tyrosine phosphorylation of Jak1, Jak2, and a slight activation of Tyk2 were reached after 15 min of G-CSF stimulation in JEG-3 cells. A similar pattern of kinase phosphorylation was obtained in G-CSF-stimulated NFS-60 cells (Fig. 2).

G-CSF activation of Jak kinases leads to tyrosine phosphorylation of STAT proteins, which form homodimers or heterodimers before translocation to the nucleus, where they activate specific target genes [Nicholson et al., 1995; Avalos, 1996; de Koning et al., 1996; Tian et al., 1996; Shimoda et al., 1997; Ward et al., 1999; Akbarzadeh and Layton, 2001]. After incubating cells with G-CSF for 15 min, STAT1 and STAT3 immunoprecipitates from unstimulated and G-CSF stimulated JEG-3 and NFS-60 cells were probed with anti-PY. As shown in Figure 3, G-CSF induced the tyrosine phosphorylation of STAT3, but not STAT1, in the two cell lines examined, being the phosphorylation level obtained in JEG-3 cells lower than that observed in NFS-60 cells.

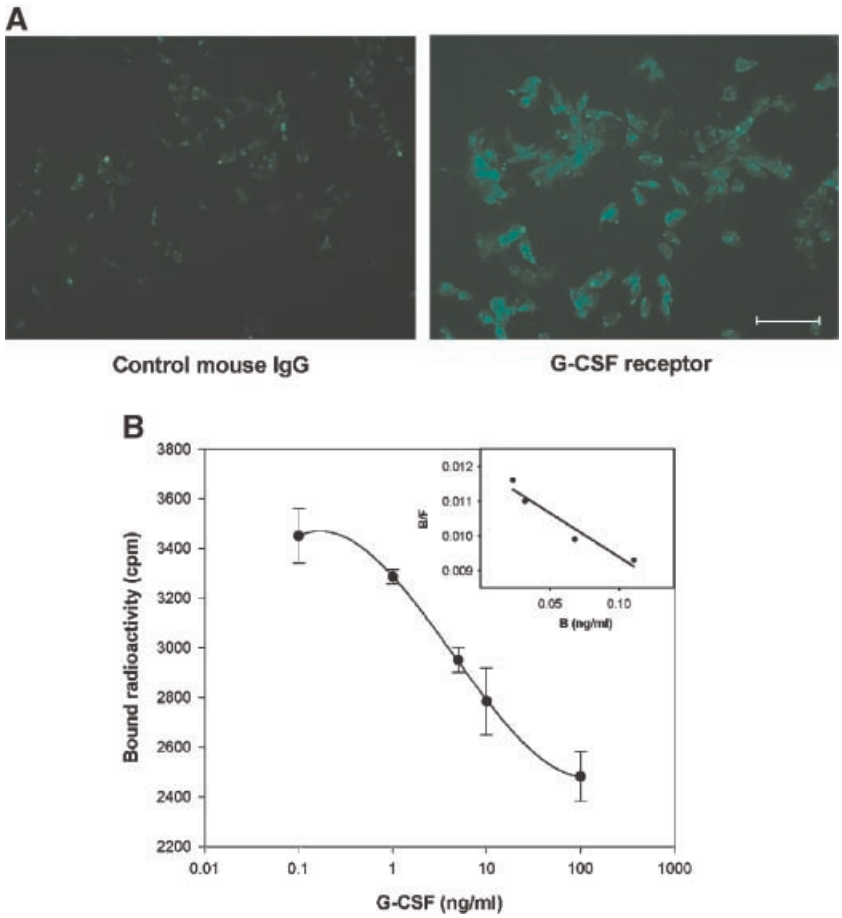

Fig. 1. Detection of G-CSF receptors in JEG-3 cells. **A**: JEG-3 cells were grown on coverslips, fixed with 4% paraformaldehyde and stained as described in Materials and Methods with a monoclonal antibody anti-G-CSF receptor or normal mouse IgG (control). Scale bar corresponds to 50 μm. **B**: Monolayers with $\sim 7 \times 10^5$ JEG-3 cells/well were incubated for 4 h at 4°C with 300,000 c.p.m. of [^{125}I]G-CSF and different concentrations of unlabeled cytokine under the conditions described in Materials and Methods. Results from one representative experiment are shown. Values are mean ± SD of three determinations. Scatchard analysis of binding data is showed in the inset. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

G-CSF Stimulates MAP Kinase Phosphorylation in JEG-3 Cells

MAP kinase signaling pathway regulates the proliferation, differentiation, and survival of hematopoietic cells [Bashey et al., 1994; Nicholson et al., 1995; Avalos, 1996; Rausch and Marshall, 1999; Suzuki et al., 1999; Akbarzadeh and Layton, 2001; Koay et al., 2002; Miranda et al., 2002; Srinivasa and Doshi, 2002]. To examine whether G-CSF activates this pathway in JEG-3 cells, phosphorylation kinetics of p38 and p44/42 MAPK were evaluated by Western blot analyses with phospho-specific antibodies. Phosphorylation of p38 in serum-deprived JEG-3 cells was observed after 15 min of stimulation with G-CSF and remained elevated for at least 120 min poststimulation (Fig. 4A). p38 activation was also evident in NFS-60 cells after 30 min of G-CSF stimulation (Fig. 4A). When JEG-3 cells were pretreated with the specific p38 MAPK inhibitor, SB203580 [Young et al., 1997], a decrease in cytokine-induced p38 MAPK phosphorylation was observed (Fig. 4B).

G-CSF also induced the phosphorylation of p44/42 MAPK in JEG-3 cells. Phosphorylation levels reached a peak after 30 min and declined gradually thereafter (Fig. 5A). Similarly, p44/42 was phosphorylated in NFS-60 cells after 30 min of incubation (Fig. 5A). The addition of PD98059, a p44/42 MAPK kinase inhibitor [Dudley et al., 1995], to trophoblastic cells strongly inhibited G-CSF-induced p44/42 MAPK phosphorylation (Fig. 5B).

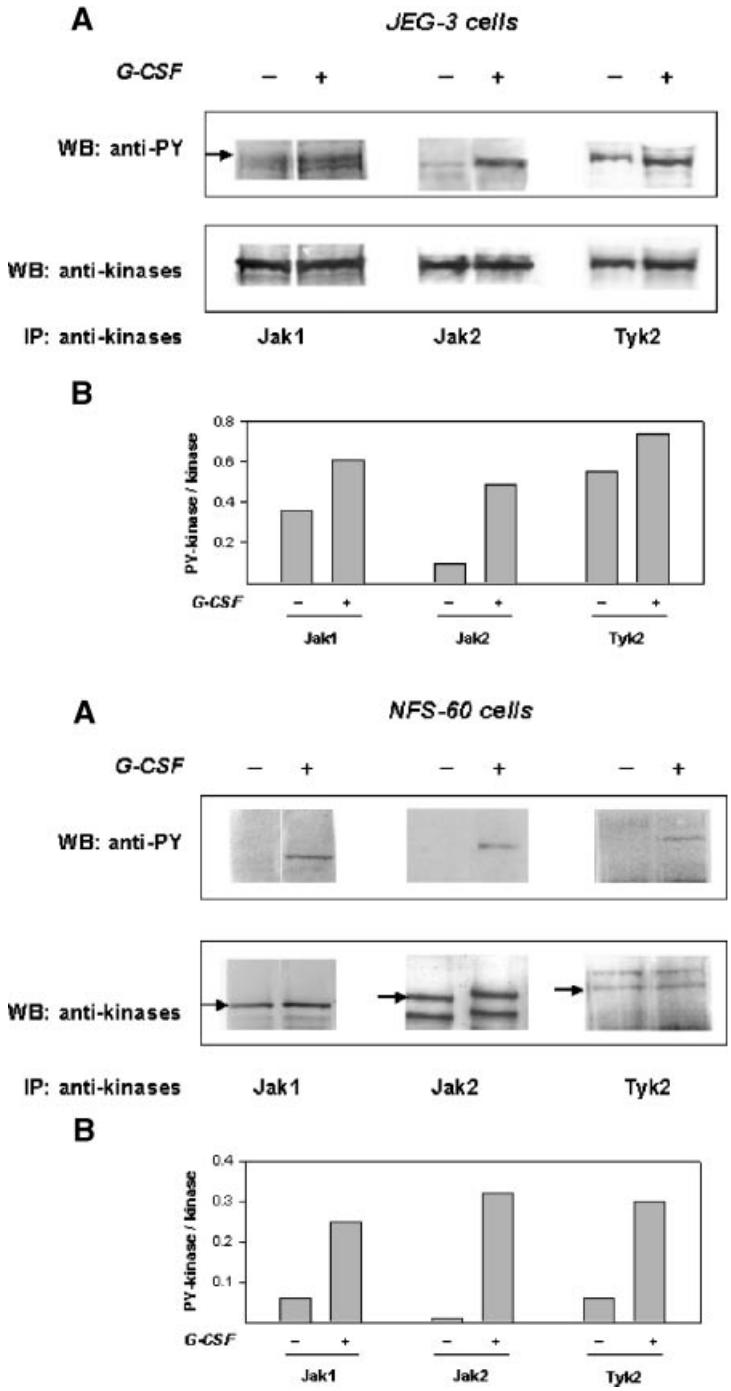

Fig. 2. Janus kinases phosphorylation by G-CSF. JEG-3 cells (**upper panel**) and NFS-60 cells (**lower panel**) were incubated for 15 min in the absence or presence of 1 or 0.1 μg/ml of G-CSF, respectively, under the conditions described in Materials and Methods. **A**: Cells were lysed and then incubated with antisera against Jak1, Jak2, and Tyk2 and the immunoprecipitates were analyzed by Western blotting with an anti-phosphotyrosine antibody (anti-PY). Membranes were stripped and reprobed with the same anti-kinase antibody employed for immunoprecipitation. Results from one representative experiment are shown. **B**: Quantification of tyrosine phosphorylation by densitometric analysis. IP, immunoprecipitation; WB, Western blot.

Effect of G-CSF on JEG-3 Cell Viability

G-CSF induces a proliferative response in NFS-60 cells [Hara et al., 1988; Marino et al., 2003]. Under our experimental conditions, NFS-60 cell growth increased in a dose-dependent manner up to a concentration of 0.1 ng/ml of G-CSF (data not shown). On the contrary, no

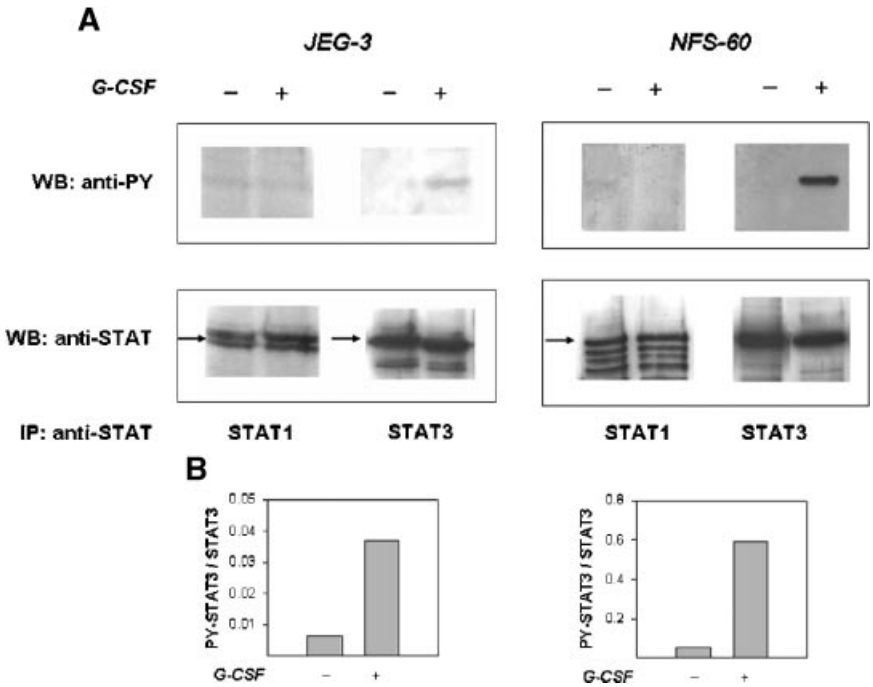

Fig. 3. STATs activation by G-CSF. JEG-3 or NFS-60 cells were incubated for 15 min in the absence or presence of 1 or 0.1 μg/ml of G-CSF, respectively, as described in Materials and Methods. **A**: Cells lysates were immunoprecipitated with antisera against STAT1 and STAT3 and then analyzed by Western blotting with an anti-phosphotyrosine antibody (anti-PY). Membranes were stripped and reprobed with the same anti-STAT antibody employed for immunoprecipitation. Results from one representative experiment are shown. **B**: Quantification of tyrosine phosphorylation by densitometric analysis. IP, immunoprecipitation; WB, Western blot.

proliferative effect was observed after incubating JEG-3 cells in the presence of 10% of FBS with concentrations of G-CSF up to 1 μg/ml (Fig. 6). However, when cells were deprived of FBS and incubated with G-CSF for 72 h, a protective effect of G-CSF on trophoblastic cells was revealed. Thus, as shown in Figure 6, cell viability diminished almost 40% under serum deprivation, while G-CSF treatment increased the number of viable cells.

To examine whether G-CSF induced an antiapoptotic effect, JEG-3 cells were incubated in the absence of serum at different times with or without G-CSF and the sub-G1 population of cells, which represents an apoptotic state, was evaluated by flow cytometry. As shown in Figure 7, although a progressive increase in the percentage of hypodiploid cells was observed under serum starvation, G-CSF stimulus significantly diminished the sub-G1 cell population after 24 and 48 h of incubation. Results derived from three independent experiments performed at 48 h showed that the amount of hypodiploid cells decreased $42 \pm 6\%$ after G-CSF treatment.

DISCUSSION

G-CSF and its receptor play an essential role in the regulation of hematopoiesis, although other functions have also been explored in other non-hematopoietic cells. Thus, it has been recently demonstrated that G-CSF increases the expression of adhesion receptors on endothelial cells [Bussolino et al., 1989], promotes survival of cardiomyocytes [Harada et al., 2005] and stimulates neurogenesis [Jung et al., 2006]. In addition, a potential role in the regulation of placental growth and specifically in trophoblast function during placentation has been suggested [Shorter et al., 1992; Mc Cracken et al., 1996, 1999]. Similarly, other cytokines produced by the maternal endometrium and the developing embryo have been connected with the implantation process and could influence the migration, proliferation and invasion of trophoblastic cells [Qiu et al., 2004; Fitzgerald et al., 2005; Pongcharoen et al., 2006].

Although G-CSFR has been found in foetal trophoblast [Uzumaki et al., 1989; Mc Cracken et al., 1996, 1999], the intracellular signals

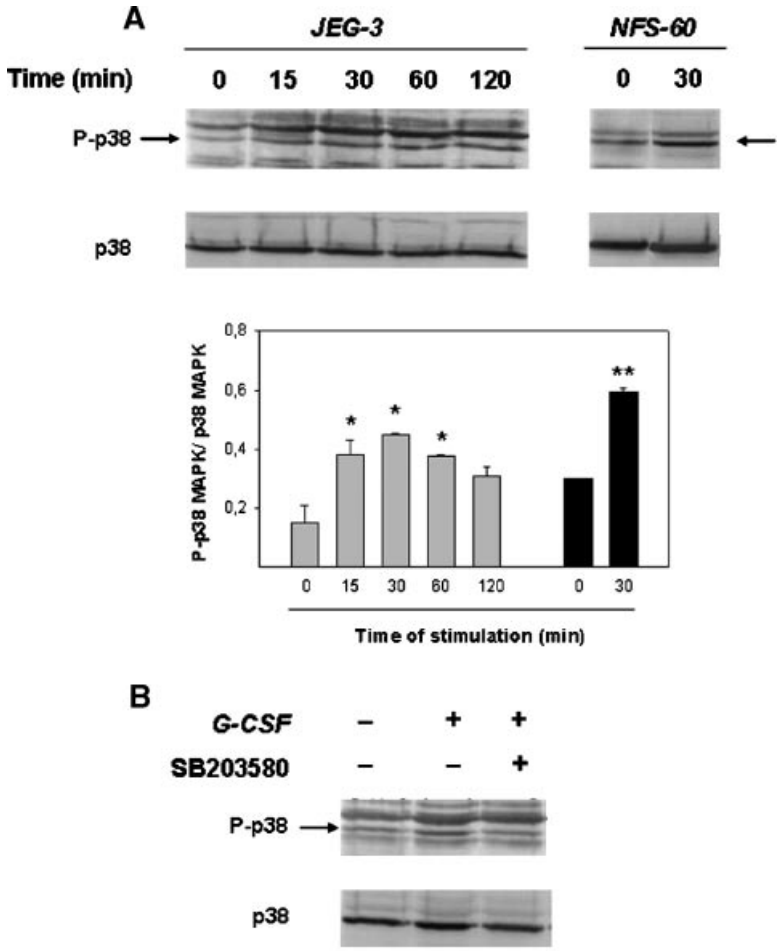

Fig. 4. Phosphorylation of p38 MAPK by G-CSF. **A**: JEG-3 and NFS-60 cells were incubated in the absence or presence of 1 μg/ml of G-CSF for the indicated times and then cell lysates were subjected to SDS–PAGE under the conditions described in Materials and Methods. Western blot assays were performed with anti-phospho-p38 MAPK antibody (P-p38) and anti-p38 antibody (p38). Results from one representative experiment are shown. Data quantification for JEG-3 (gray bars) and NFS-60 cells (black bars) was performed by densitometric analysis. Results represent mean values ± SE of three different experiments (*$P < 0.05$, **$P < 0.001$). **B**: JEG-3 cells were pretreated for 30 min with or without 10 μM SB203580 and after stimulating 30 min with 1 μg/ml G-CSF, p38 MAPK phosphorylation was studied by immunoblot.

triggered after G-CSF stimulation have not been examined. In the present paper, we demonstrated the presence of specific G-CSFR in JEG-3 cells, a human choriocarcinoma cell line that exhibits a biochemical behavior similar to that of the first trimester trophoblast cells [Kohler et al., 1971; Mandl et al., 2002; Karmakar et al., 2004]. We showed that G-CSF activates Jak1, Jak2, Tyk2, and STAT3, but not STAT1 phosphorylation both in JEG-3 cells as well as in NFS-60 myeloid cells that express a G-CSF functional receptor.

The Jak/STAT pathway has been widely recognized in hematopoietic cells, where it contributes to regulate the G-CSF-induced proliferation, differentiation and survival [Demetri and Griffin, 1991; Avalos, 1996; Akbarzadeh and Layton, 2001]. In particular, STAT3 activation has been correlated with both differentiation and survival of myeloid cells [Shimozaki et al., 1997; Ward et al., 1999; de Koning et al., 2000; Smithgall et al., 2000; McLemore et al., 2001]. More recently, it has been reported that STAT3 would be directly involved in the antiapoptotic effect exerted by G-CSF in cardiomyocytes, a cell type that does not proliferate in the presence of G-CSF [Harada et al., 2005].

The MAPK pathway also plays a role in the regulation of cell growth and survival of hematopoietic cells. Thus, activation of p38 MAPK pathway has been shown to be necessary for

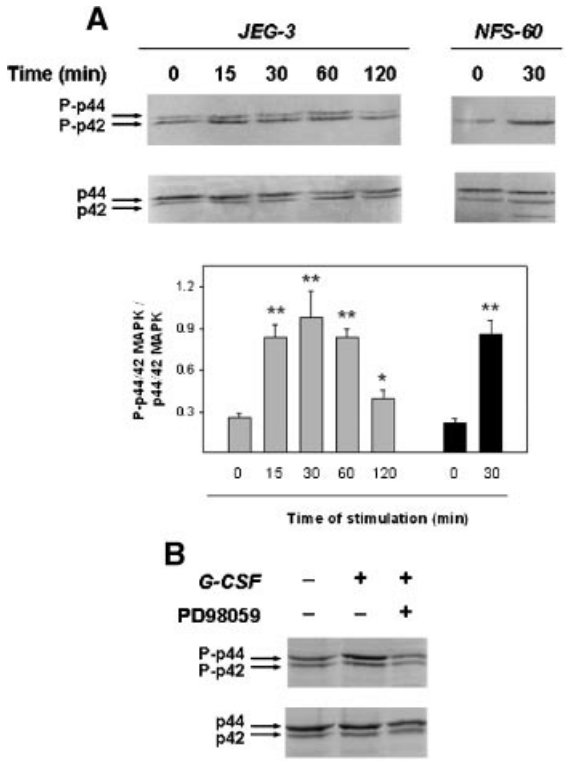

Fig. 5. Phosphorylation of p44/42 MAPK by G-CSF. **A**: After incubating JEG-3 and NFS-60 cells with or without 1 μg/ml of G-CSF for the indicated times, cell lysates were subjected to SDS–PAGE and Western blot with anti-phospho-p44/42 MAPK antibody (P-p44/42) and anti-p44/42 antibody (p44/42). Results from one representative experiment are shown. Data quantification for JEG-3 (gray bars) and NFS-60 cells (black bars) was performed by densitometric analysis. Results represent mean values ± SE of three different experiments (*$P < 0.05$, **$P < 0.001$). **B**: The inhibition of cytokine-induced p44/42 MAPK activation was studied after treating JEG-3 cells for 30 min in the absence or presence of 1 μM PD98059 inhibitor and incubating 30 min with 1 μg/ml G-CSF.

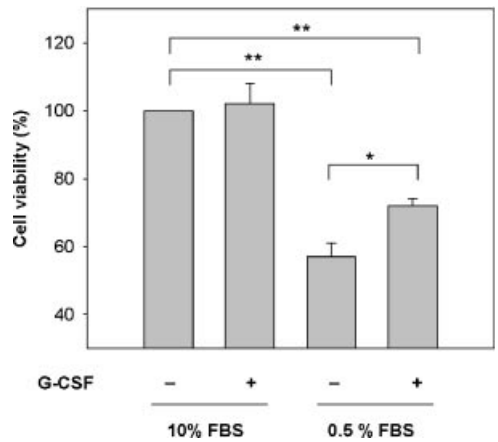

Fig. 6. G-CSF effect on JEG-3 cell viability. JEG-3 cells (2.5×10^5 cell/ml) were incubated for 72 h at 37°C in 96-well culture microplates in the presence or absence of 1 μg/ml G-CSF in a total volume of 100 μl of medium containing 10% or 0.5% FBS as described in Materials and Methods. Cell viability was evaluated by MTS assay. Results are expressed as the percentage of cell viability obtained in complete culture medium and represent the mean ± SE of three independent experiments. Statistical analyses were performed by Student's *t*-test and are indicated by *$P < 0.01$ and **$P < 0.005$.

G-CSF-induced hematopoietic cell proliferation [Rausch and Marshall, 1999; Srinivasa and Doshi, 2002], although this pathway would not be involved in mature neutrophils functions [Suzuki et al., 1999]. In addition, whereas some authors demonstrate a participation of this pathway in the granulocyte apoptotic cell death [Frasch et al., 1998; Aoshiba et al., 1999], others claim an essential role for p38 MAPK in granulocyte survival [Villunger et al., 2000]. When this pathway was evaluated in trophoblastic JEG-3 cells, we found that G-CSF induced p38 phosphorylation in a time-dependent manner. Particularly, in placenta tissues, p38MAPK has been associated to a normal trophoblast development and placental angiogenesis [Mudgett et al., 2000]. It has also been proposed that this cascade would be required to increase the G-CSF-induced expression of adhesion receptors on endothelial cells [Bussolino et al., 1989]. Based on these findings, it would be valuable to examine a possible role of G-CSF-induced p38 MAPK pathway in the decidual invasion of trophoblastic cells.

We also demonstrated the activation of p44/42 MAPK pathway both in JEG-3 and NFS-60 cells. This cascade has been previously associated to G-CSF-induced proliferation [Bashey et al., 1994; Rausch and Marshall, 1999; Srinivasa and Doshi, 2002; Koay et al., 2002], myeloid differentiation [Miranda et al., 2002] and regulation of survival in hematopoietic cells [Dent et al., 1998; Miranda et al., 2002], although the response triggered by p44/42 MAPK activation after G-CSF stimulus in trophoblastic cells remains unknown.

In the present study, when JEG-3 cell response to G-CSF was evaluated, it was demonstrated that G-CSF did not stimulate cell growth, although a cytokine protective effect was evident. Thus, a higher viability and a decrease in the population of apoptotic cells were obtained after G-CSF treatment. Our results also showed that G-CSF activates similar pathways both in JEG-3 cells that exhibit a nonproliferative response to G-CSF, as well as in the proliferative NFS-60 cell line,

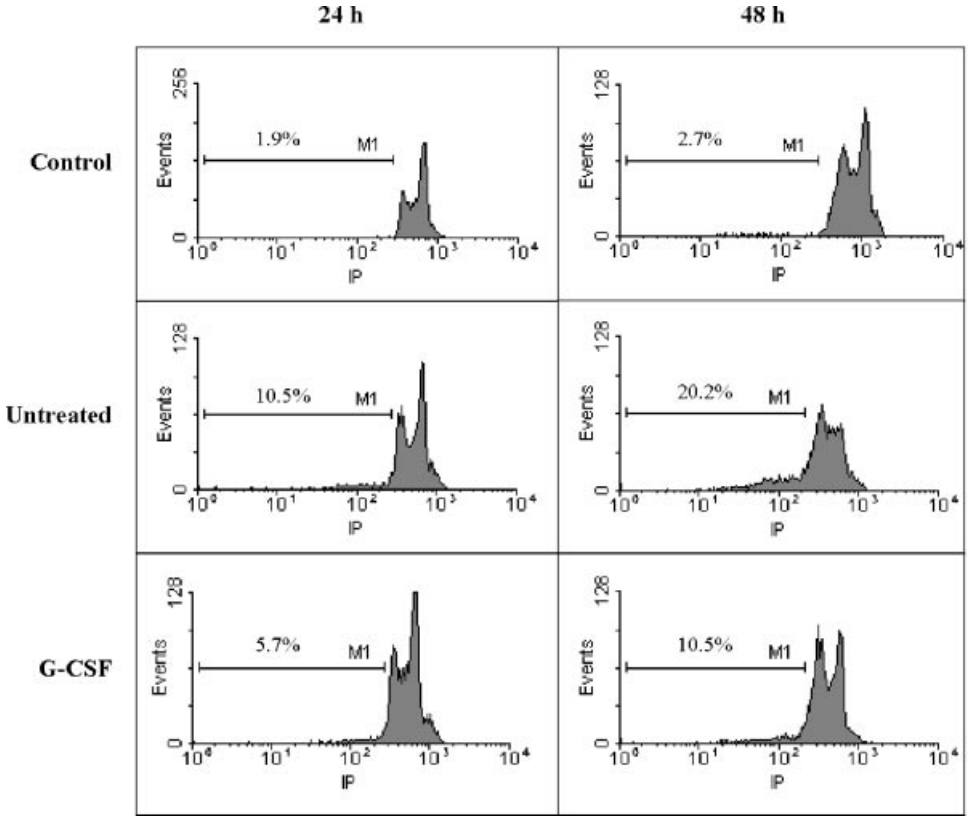

Fig. 7. Effect of G-CSF on the sub-G1 population of JEG-3 cells. Cells were incubated for 24 or 48 h in complete culture medium (control) or under serum starvation in the absence (untreated) or presence of 1 μg/ml G-CSF. DNA content was then evaluated after propidium iodide staining by a FACScan flow cytometer. Histograms from one representative experiment are shown.

indicating a different functional role of these cascades according to the cell type examined.

In summary, this study represents the first report of the presence of a functional G-CSFR in trophoblastic cells, which activates intracellular signals that could be related to survival, angiogenesis, migration, or invasion. Although the relative contribution of Jak/STAT and MAPK pathways on these processes remains to be studied, our results demonstrated an antiapoptotic role of G-CSF on JEG-3 cells that could be involved in placenta development.

ACKNOWLEDGMENTS

We are indebted to Dr Viviana C. Blank (IQUIFIB, Buenos Aires, Argentina) for helpful discussion and critical revision of the manuscript. This work was supported by grants from CONICET, FONCYT and Universidad de Buenos Aires, Argentina.

REFERENCES

Akbarzadeh S, Layton JE. 2001. Granulocyte colony-stimulating factor receptor: Structure and function. Vitam Horm 63:159–194.

Aoshiba K, Yasui S, Hayashi M, Tamaoki J, Nagai A. 1999. Role of p38-mitogen-activated protein kinase in spontaneous apoptosis of human neutrophils. J Immunol 162:1692–1700.

Avalos BR. 1996. Molecular analysis of the granulocyte colony-stimulating factor receptor. Blood 88:761–777.

Bahn RS, Worsham A, Speeg KV, Ascoli M, Rabin D. 1981. Characterization of steroid production in cultured human choriocarcinoma cells. J Clin Endocrinol Metab 52:447–450.

Bashey A, Healy L, Marshall CJ. 1994. Proliferative but not nonproliferative responses to granulocyte colony-stimulating factor are associated with rapid activation of the p21ras/MAP kinase signalling pathway. Blood 83:949–957.

Begley CG, Metcalf D, Nicola NA. 1988. Binding characteristics and proliferative action of purified granulocyte-colony stimulating factor (G-CSF) on normal and leukemic human promyelocytes. Exp Hematol 16:71–79.

Bussolino F, Wang JM, Defilippi P, Turrini F, Sanavio F, Edgell CJ, Aglietta M, Arese P, Mantovani A. 1989.

Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. Nature 337:471–473.

Cory AH, Owen TC, Barltrop JA, Cory JG. 1991. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun 3:207–212.

de Koning JP, Dong F, Smith L, Schelen AM, Barge RM, van der Plas DC, Hoefsloot LH, Lowenberg B, Touw IP. 1996. The membrane-distal cytoplasmic region of human granulocyte colony-stimulating factor receptor is required for STAT3 but not STAT1 homodimer formation. Blood 87:1335–1342.

de Koning JP, Soede-Bobok AA, Ward AC, Schelen AM, Antonissen C, van Leeuwen D, Lowenberg B, Touw IP. 2000. STAT3-mediated differentiation and survival and of myeloid cells in response to granulocyte colony-stimulating factor: Role for the cyclin-dependent kinase inhibitor p27 (Kip1). Oncogene 19:3290–3298.

Demetri GD, Griffin JD. 1991. Granulocyte colony-stimulating factor and its receptor. Blood 78:2791–2808.

Dent P, Jarvis WD, Birrer MJ, Fisher PB, Schmidt-Ullrich RK, Grant S. 1998. The roles of signaling by the p42/p44 mitogen-activated protein (MAP) kinase pathway; a potential route to radio- and chemo-sensitization of tumor cells resulting in the induction of apoptosis and loss of clonogenicity. Leukemia 12:1843–1850.

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 92: 7686–7689.

Fitzgerald JS, Busch S, Wengenmayer T, Foerster K, de la Motte T, Poehlmann TG, Markert UR. 2005. Signal transduction in trophoblast invasion. Chem Immunol Allergy 88:181–199.

Frasch SC, Nick JA, Fadok VA, Bratton DL, Worthen GS, Henson PM. 1998. p38 mitogen-activated protein kinase-dependent and -independent intracellular signal transduction pathways leading to apoptosis in human neutrophils. J Biol Chem 273:8389–8397.

Fuste B, Mazzara R, Escolar G, Merino A, Ordinas A, Diaz-Ricart M. 2004. Granulocyte colony-stimulating factor increases expression of adhesion receptors on endothelial cells through activation of p38 MAPK. Haematologica 89:578–585.

Handa A, Kashimura T, Takeuchi S, Yamamoto A, Murohashi I, Bessho M, Hirashima K. 2000. Expression of functional granulocyte-colony stimulating factor receptors on human B-lymphocytic leukaemia cells. Ann Hematol 79:127–131.

Hara K, Suda T, Suda J, Eguchi M, Ihle JN, Nagata S, Miura Y, Saito M. 1988. Bipotential murine hemopoietic cell line (NFS-60) that is responsive to IL-3, GM-CSF, G-CSF, and erythropoietin. Exp Hematol 16:256–261.

Harada M, Qin Y, Takano H, Minamino T, Zou Y, Toko H, Ohtsuka M, Matsuura K, Sano M, Nishi J, Iwanaga K, Akazawa H, Kunieda T, Zhu W, Hasegawa H, Kunisada K, Nagai T, Nakaya H, Yamauchi-Takihara K, Komuro I. 2005. G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. Nat Med 11:305–311.

Jung KH, Chu K, Lee ST, Kim SJ, Sinn DI, Kim SU, Kim M, Roh JK. 2006. Granulocyte colony-stimulating factor stimulates neurogenesis via vascular endothelial growth factor with STAT activation. Brain Res 1073/1074:190–201.

Karmakar S, Dhar R, Das C. 2004. Inhibition of cytotrophoblastic (JEG-3) cell invasion by interleukin 12 involves an interferon γ-mediated pathway. J Biol Chem 279:55297–55307.

Koay DC, Nguyen T, Sartorelli AC. 2002. Distinct region of the granulocyte colony-stimulating factor receptor mediates proliferative signaling through activation of Janus kinase 2 and p44/42 mitogen-activated protein kinase. Cell Signal 14:239–247.

Kohler PO, Bridson WE, Hammond JM, Weintraub B, Kirschner MA, Van Thiel DH. 1971. Clonal lines of human choriocarcinoma cells in culture. Acta Endocrinol Suppl 153:137–153.

Mandl M, Haas J, Bischof P, Nöhammer G, Desoye G. 2002. Serum-dependent effects of IGF-I and insulin on proliferation and invasion of human first trimester trophoblast cell models. Histochem Cell Biol 117:391–399.

Marino VJ, Sterin-Prync AE, Carbonetto CH, Roguin LP. 2001. Conformational and sequential epitopes on the human granulocyte-colony stimulating factor (hG-CSF) and their role in binding to human placenta receptors. Cytokine 16:41–50.

Marino VJ, Sterin-Prync AE, Roguin LP. 2003. Change in the accessibility of an epitope of the human granulocyte-colony stimulating factor (hG-CSF) after binding to receptors. Cytokine 22:5–11.

Mc Cracken SA, Cracken SA, Layton JE, Shorter SC, Starkey PM, Barlow DH, Mardon HJ. 1996. Expression of granulocyte-colony stimulating factor and its receptor is regulated during the development of the human placenta. J Endocrinol 149:249–258.

Mc Cracken SA, Grant KE, McKenzie IZ, Redman CWG, Mardon HJ. 1999. Gestational regulation of granulocyte-colony stimulating factor receptor expression in the human placenta. Biol Reprod 60:790–796.

McLemore ML, Grewal S, Liu F, Archambault A, Poursine-Laurent J, Haug J, Link DC. 2001. STAT-3 activation is required for normal G-CSF-dependent proliferation and granulocytic differentiation. Immunity 14:193–204.

Miranda MB, McGuire TF, Jonson DE. 2002. Importance of MEK-1/-2 signaling in monocytic and granulocytic differentiation of myeloid cell lines. Leukemia 16:683–692.

Morstyng G, Burgess AW. 1988. Hemopoietic growth factors: A review. Cancer Res 48:5624–5637.

Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S, Shen MM. 2000. Essential role for p38 alpha mitogen-activated protein kinase in placental angiogenesis. Proc Natl Acad Sci USA 97:10454–10459.

Nicholson SE, Oates AC, Harpur AG, Ziemiecki A, Wilks AF, Layton JE. 1994. Tyrosine kinase JAK1 is associated with the granulocyte-colony-stimulating factor receptor and both become tyrosine-phosphorylated after receptor activation. Proc Natl Acad Sci USA 91:2985–2988.

Nicholson SE, Novak U, Ziegler SF, Layton JE. 1995. Distinct regions of the granulocyte colony-stimulating factor receptor are required for tyrosine phosphorylation of the signaling molecules JAK2, Stat3, and p42, p44MAPK. Blood 86:3698–3704.

Nicola NA. 1989. Hemopoietic cell growth factors and their receptors. Annu Rev Biochem 58:45–77.

Nicola NA, Metcalf D. 1985. Binding of ^{125}I-labeled granulocyte-colony stimulating factor to normal murine hemopoietic cells. J Cell Physiol 124:313–321.

Pongcharoen S, Niumsup P, Sanguansermsri D, Supalap K, Butkhamchot P. 2006. The effect of interleukin-17 on the proliferation and invasion of JEG-3 human choriocarcinoma cells. Am J Reprod Immunol 55:291–300.

Qiu Q, Yang M, Tsang BK, Gruslin A. 2004. Both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling are required in epidermal growth factor-induced human trophoblast migration. Mol Hum Reprod 10:677–684.

Rausch O, Marshall CJ. 1999. Cooperation of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways during granulocyte colony-stimulating factor-induced hemopoietic cell proliferation. J Biol Chem 274:4096–4105.

Scatchard G. 1949. The attraction of proteins for small molecules and ions. Ann NY Acad Sci 51:660–672.

Shimoda K, Okamura S, Harada N, Ikematsu W, Kondo S, Kawasaki C, Tanaka T, Etou T, Akashi K, Okamura T, Shibuya T, Harada M, Niho Y. 1992. Granulocyte colony-stimulating factor receptors on human acute leukaemia: Biophenotypic leukemic cells posses granulocyte colony-stimulating factor receptors. Cancer Res 2:3052–3055.

Shimoda K, Feng J, Murakami H, Nagata S, Watling D, Rogers NC, Stark GR, Kerr IM, Ihle JN. 1997. Jak1 plays an essential role for receptor phosphorylation and stat activation in response to granulocyte colony-stimulating factor. Blood 90:597–604.

Shimozaki K, Nakajima K, Hirano T, Nagata S. 1997. Involvement of STAT3 in the granulocyte colony-stimulating factor-induced differentiation of myeloid cells. J Biol Chem 272:25184–25189.

Shinjo K, Takeshita A, Ohnishi K, Ohno R. 1995. Expression of granulocyte-colony stimulating factor receptor increases with differentiation in myeloid cells by a newly-devised quantitative flow-cytometric assay. Br J Hematol 91:783–794.

Shorter SC, Vince GS, Starkey PM. 1992. Production of granulocyte colony-stimulating factor at the materno-foetal interface in human pregnancy. Immunology 75: 468–474.

Smithgall TE, Briggs SD, Schreiner S, Lerner EC, Cheng H, Wilson MB. 2000. Control of myeloid differentiation and survival by Stats. Oncogene 19:2612–2618.

Srinivasa SP, Doshi PD. 2002. Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways cooperate in mediating cytokine-induced proliferation of a leukemic cell line. Leukemia 16:244–253.

Suzuki K, Hino M, Hato F, Tatsumi N, Kitagawa S. 1999. Cytokine-specific activation of distinct mitogen-activated protein kinase subtype cascades in human neutrophils stimulated by granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-alpha. Blood 93:341–349.

Tian SS, Tapley P, Sincich C, Stein RB, Rosen J, Lamb P. 1996. Multiple signaling pathways induced by granulocyte colony-stimulating factor involving activation of JAKs, STAT5, and/or STAT3 are required for regulation of three distinct classes of immediate early genes. Blood 88:4435–4444.

Uzumaki H, Okabe T, Sasaki N, Hagiwara K, Takaku F, Tobita M, Yasubawa K, Ito S, Umezawa Y. 1989. Identification and characterization of receptors for granulocyte colony-stimulating factor on human placenta and trophoblastic cells. Proc Natl Acad Sci USA 86:9323–9326.

Villunger A, O'Reilly LA, Holler N, Adams J, Strasser A. 2000. Fas ligand, Bcl-2, granulocyte colony-stimulating factor, and p38 mitogen-activated protein kinase: Regulators of distinct cell death and survival pathways in granulocytes. J Exp Med 192:647–658.

Ward AC, Smith L, de Koning JP, van Aesch Y, Touw IP. 1999. Multiple signals mediate proliferation, differentiation, and survival from the granulocyte colony-stimulating factor receptor in myeloid 32D cells. J Biol Chem 274:14956–14962.

Young P, McLaughlin M, Kumar S, Kassis S, Doyle M, McNulty D, Gallagher T, Fisher S, McDonnell P, Carr S, Huddleston M, Seibel G, Porter T, Livi G, Adams J, Lee J. 1997. Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. J Biol Chem 272:12116–12121.