

Analysis of antiapoptosis activity of human GM-CSF receptor

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Human GM-CSF (hGM-CSF) induces proliferation and sustains the viability of a mouse IL-3-dependent lymphoid cell line BA/F3 that expresses the functional hGM-CSF receptor (hGMR). To reveal an antiapoptotic mechanism of hGM-CSF, we analyzed various apoptotic markers of BA/F3 cells in various conditions. Within 24 hours of factor depletion, caspase 3-like, but not caspase 1-like, enzyme activity and DNA fragmentation were augmented. Analysis with the tyrosine kinase inhibitor (genistein) and an MEK1 inhibitor (PD98059) on antiapoptosis activity indicates that the activation of either the genistein-sensitive signaling pathway or the PD98059-sensitive signaling pathway of the β c subunit may be sufficient to suppress apoptosis through hGMR. Because hGMR mutants (which activate JAK2 but neither STAT5 nor the MAPK cascade) have antiapoptotic activity in BA/F3 cells, the involvement of JAK2, excluding the molecules mentioned earlier, for antiapoptosis activity seems likely. Because the JAK2 inhibitor AG-490 suppressed the antiapoptotic activity of hGM-CSF, the essential role for JAK2 activation to maintain the viability is considered. Interestingly, hGMR mutants, which lack MAPK cascade activation, require a higher dose of hGM-CSF than that for wild-type hGMR. Because the expression level and affinity to hGM-CSF among wild-type hGMR and mutant hGMR are the same, we speculated that biologic response is determined by a combination of strength of various signaling events. (*J Allergy Clin Immunol* 2000;106:S10-18.)

Key words: Cytokine receptor, signal transduction, JAK2, apoptosis

The receptors of IL-3 and GM-CSF (GMR) consist of 2 subunits (α and β), both of which are members of the cytokine receptor superfamily.¹⁻³ The α subunit is specific for each cytokine, although the common β subunit (β c) is shared by receptors for IL-3, GM-CSF, and IL-5.² Although tyrosine phosphorylation of the β c and several cytoplasmic proteins occur after stimulation of IL-3 or GM-CSF,⁴⁻⁶ the cytokine receptor family (including the IL-3 receptor and GMR) does not contain a kinase domain or activity in the receptor itself, and Janus kinase 2 (JAK2) plays a primary role in IL-3 or GM-CSF signaling.^{7,8} Using dominant negative JAK2, we showed that JAK2 plays an essential role in GM-CSF-induced proliferation, the induction of immediate early genes and the phosphorylation of cellular proteins and β c itself.⁹

Abbreviations used

β c:	Common β subunit
GMR:	GM-CSF receptor
hGM-CSF:	Human GM-CSF
JAK2:	Janus kinase 2
mIL-3:	Mouse IL-3
PI-3K:	Phosphatidylinositol-3-OH kinase
TUNEL:	TdT-mediated dUTP-biotin nick end labeling

We then analyzed the signaling pathway of human GM-CSF (hGM-CSF) using various β c mutants.¹⁰⁻¹³ The β c has conserved box1 and box2 regions and 8 tyrosine residues in the cytoplasmic domain.¹⁴ We analyzed various mutants of β c^{10,15,16} in BA/F3 cells and NIH3T3 cells and found that multiple signaling pathways are activated by the hGMR. Among the mutants, we found that the Fall mutant, in which all the tyrosine residues of β c were converted to phenylalanine, was unique because cell survival could be sustained, yet cell proliferation was not activated.¹⁶ Mutants that have any 1 of the tyrosine residues of β c restored the proliferative effect, which indicated the role of tyrosine residues for proliferation but not for the survival of BA/F3 cells. Thus, Fall is an excellent model system to elucidate cell proliferation and survival signals.

Withdrawal of IL-3 from progenitor cell lines or primary IL-3-dependent cells from bone marrow resulted in apoptosis of the cells.¹⁷⁻¹⁹ In several mouse IL-3- (mIL-3) or hGM-CSF-dependent cell lines such as 32D and BA/F3 cells, apoptosis was triggered by factor depletion. Exposing mIL-3-dependent BA/F3 cells²⁰ to γ irradiation induced apoptosis more rapidly than that seen by factor depletion of the same cell line.^{21,22} The involvement of various molecules for IL-3- or GM-CSF-induced antiapoptosis has been reported. Bcl-2 protein is an inner mitochondrial membrane protein,²³ and its overexpression prolongs the survival of hematopoietic cells.^{19,24,25} The role of Bcl-x_L²⁶ for antiapoptosis has been suggested. The potential of IL-3 to regulate the expression level of these proteins was also reported.²⁷ The roles of phosphatidylinositol-3-OH kinase (PI-3K), Akt, a serine-threonine protein kinase, and the Bad is Bad (BAD) pathway have been characterized in several systems, including IL-3 signaling.²⁸ BAD, which forms heterodimer with Bcl-x_L or Bcl-2 and neutralizes their protective effects and promotes cell death,²⁹ can be regulated by IL-3 through a cascade of activation of PI-3K and Akt.³⁰⁻³² Active Ras binds to PI-3K,³³ but it is still not clear whether PI-3K lies down-

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stream of *ras* with regard to cytokine signals. One report that used dominant negative MAPK kinase suggested a role of the MAPK cascade in IL-3-induced *bcl-x* gene expression,²⁷ and it was reported that the MEK1 inhibitor PD98059 suppressed the viability of BA/F3 cells that were dependent on the active form of Ras, which also binds with Bcl-2.^{34,35}

The present study was focused on the analysis of signals that are related to cell survival by IL-3 or GM-CSF. We found an essential role of JAK2 through box1 region of βc to prevent apoptosis. It became apparent that there are multiple pathways to sustain survival of BA/F3 cells through hGM-CSF receptors.

METHODS

Chemicals, media, and cytokines

FCS was purchased from Biocell Laboratories Co Ltd. RPMI 1640 was obtained from Nikken BioMedical Laboratories Co Ltd. Recombinant mIL-3 expressed in silkworm, *Bombyx mori*, was purified as described elsewhere.³⁶ Recombinant hGM-CSF and G418 were gifts from Schering-Plough Corp. Peptides and peptides conjugated with fluorescent compounds were purchased from Peptide Institute Inc (Osaka, Japan). Peptide caspase inhibitor A-Asp-Glu-Val-Asp-CH₂F was obtained from Kamiya Biomedical Company (Seattle, Wash). PD98059 was obtained from New England Biolabs, Inc. AG490 was a gift from Mitsubishi Chemical Corporation (Tokyo, Japan). Wortmannin was obtained from Sigma (Steinheim, Germany), and Genistein was purchased from Wako (Osaka, Japan).

Cell lines and culture methods

An mIL-3-dependent pro-B cell line, BA/F3,²⁰ was maintained in RPMI 1640 medium that contained 5% FCS, 0.25 ng/mL mIL-3, 100 units/mL penicillin, and 100 μ g/mL streptomycin. For cell washing or depletion, the same medium without mIL-3 was used (depletion medium). Various BA/F3 cell clones that expressed wild-type hGMR α alone (BA/F- α), wild-type hGMR α and hGMR β (BA/F-wild), or wild-type hGMR α and one of hGMR β mutants, Fall, 544, 517 and Δ box1 (BA/F-Fall, -544, -517, and - Δ box1, respectively) were grown in the same type of medium with 500 μ g/mL of G418 added as a supplement.

Analysis of caspase enzymatic activity

Cells (2×10^6) were washed with PBS and lysed in 100 μ L of lysis buffer (50 mmol/L PIPES-NaOH, pH 7.0, 50 mmol/L KCl, 5 mmol/L EGTA, 2 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride) by freezing and thawing 3 times in liquid nitrogen. Cell lysates were centrifuged at 12,000g for 5 minutes, and 10 μ L of supernatant was incubated with fluorogenic substrate peptides (final concentration at 1.5 μ mol/L) in 2 mL of enzyme reaction buffer (100 mmol/L HEPES-KOH, pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate (CHAPS), 10 mmol/L dithiothreitol, 0.1 mg/mL ovalbumin) for 30 minutes at 30°C. The peptides conjugated with fluorescent compounds, Ac-YVAD-MCA,³⁷ MocAc-YVADA PK(Dnp)-NH₂,³⁸ Ac-DEVD-MCA, and MocAc-DEVD PK(Dnp)-NH₂³⁸ were used as specific substrates for enzyme analysis of caspase 1 (YVAD) and caspase 3 (DEVD). Protease activities were determined by monitoring the release of fluorescent compounds, with the use of a spectrofluorophotometer (RF-5300PC; Shimadzu). Wavelengths are excitation wavelength of 325 nm and emission wavelength of 392 nm for Dnp and excitation wavelength 380 nm, emission wavelength 460 nm for MCA with a band path of

5 nm. Protein concentration of the lysates was determined with the BCA protein assay kit (Pierce, Rockford, Ill), and fluorescence values were expressed as relative value to the protein concentration.

DNA fragmentation assay

DNA fragmentation assay was performed by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method³⁹ and DNA ladder assay. The TUNEL assay was performed with Takara in situ apoptosis detection kits (Takara Biomedicals) according to the manufacturer's instruction. Briefly, cells ($1-3 \times 10^6$) were washed with PBS and fixed with 10% of formalin in PBS and further incubated with 0.3% H₂O₂/methanol. The cells were then permeabilized and labeled with TdT-mediated dUTP-FITC HRP conjugate and analyzed by FACSscan (Becton Dickinson, San Jose, Calif). A total of 10,000 events were collected, and ungated data are presented in all figures shown. For DNA ladder assay, cells ($1-5 \times 10^6$) were washed with PBS and lysed with 450 μ L of lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, 10 mmol/L NaCl, 0.5% [vol/vol] Triton X-100) and kept on ice for 30 minutes. The lysate was centrifuged, and the supernatant was extracted twice with phenol/chloroform (1:1); chromosomal DNA was ethanol precipitated. The DNA pellets were suspended in 20 μ L Tris-EDTA, pH 7.8, containing 20 μ g/mL RNase and electrophoresed through a 1.5% agarose gel; the fragmented DNA was stained with ethidium bromide.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as described elsewhere.^{9,10} Briefly, cells (1×10^7) were depleted of mIL-3 for 5 hours then restimulated with hGM-CSF (10 ng/mL) for the indicated duration. In some cases, the cells were precultured with inhibitors: 15 minutes for genistein (20 μ g/mL) and PD98059 (100 μ mol/L), 2 hours for AG490 (20-200 μ mol/L) before hGM-CSF stimulation. The cells were lysed with 500 μ L of lysis buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP-40, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride) for 1 hour. The immunoprecipitation of JAK2 was performed by the use of polyclonal antibody anti-JAK2 (c-20), all purchased from Santa Cruz, and protein A-sepharose (Pharmacia Biotech Inc) for 2 hours. The precipitate was analyzed by immunoblotting as described previously.^{9,10} Activation of JAK2 was examined by phosphorylation of JAK2 by Western blotting with the use of anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Incorporated). For α -fodrin Western blotting, 20 μ g protein of total cell lysate was separated through 5% SDS-PAGE. Western blotting was performed with the use of the mouse anti-spectrin monoclonal antibody (Chemicon International Inc).

RESULTS

DNA fragmentation and caspase 3, but not caspase 1, activation were enhanced in the absence of mIL-3 or hGM-CSF in BA/F3 cells

It is well documented that various cellular events, such as DNA fragmentation and caspases activation, are hallmarks of apoptosis, as induced by growth factor depletion or DNA damage in cells, including hematopoietic cells. We first characterized the factor depletion-induced DNA fragmentation by TUNEL assay³⁹ using a flow cytometer. As shown in Fig 1, A, DNA fragmentation became evident 4 hours after factor depletion and more than 80% of the cells showed DNA fragmentation after 24 hours. We also examined the apoptotic cells by staining cell surface phosphatidylserine, using fluorescein-

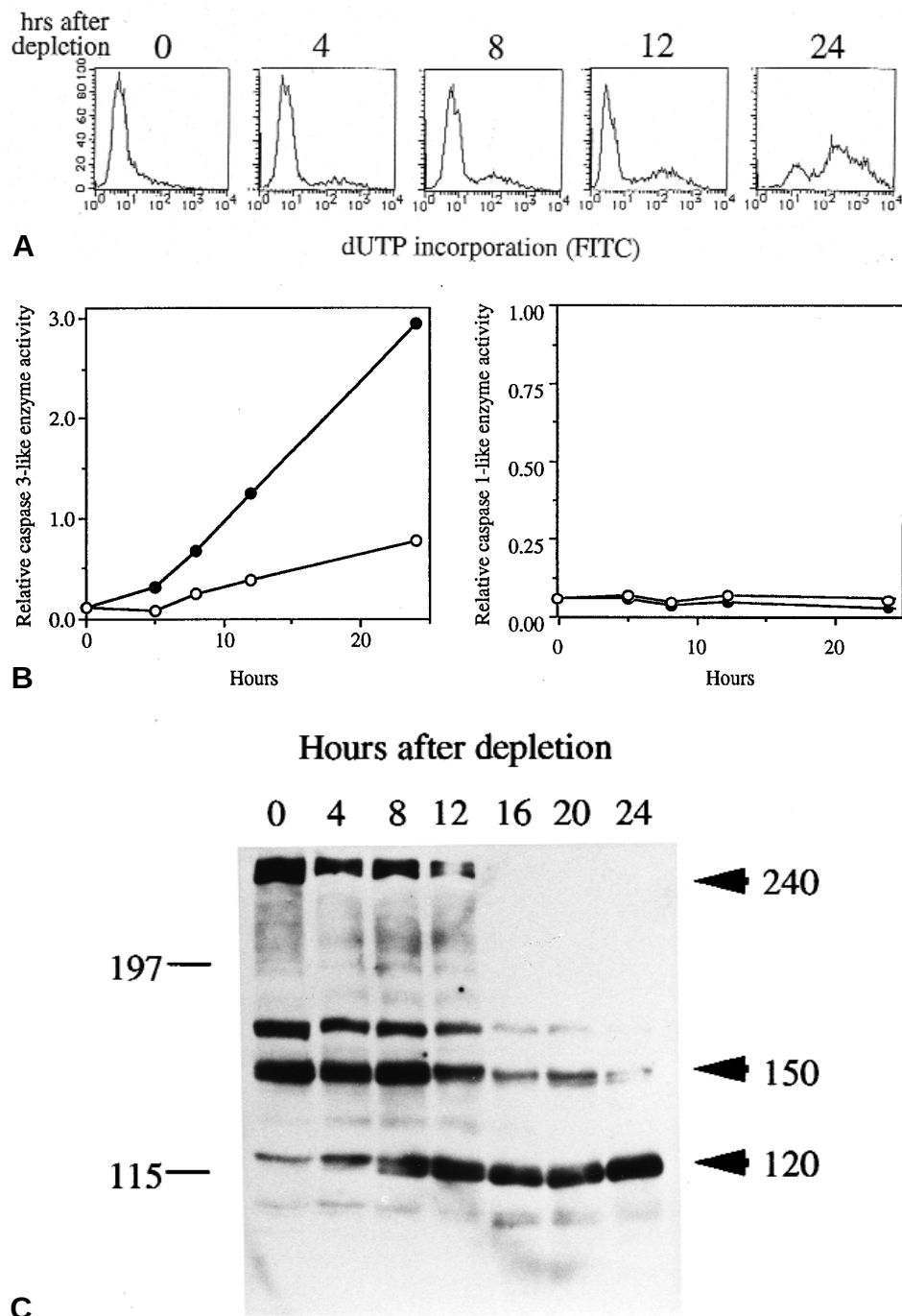


FIG 1. The induction of various apoptosis hallmarks in BA/F3 cells after mIL-3 depletion. **A**, Time course of DNA fragmentation in BA/F3 cells after removal of mIL-3. DNA fragmentation was determined by TUNEL analysis. **B**, Activities of caspases after depletion of mIL-3 in BA/F3 cells. The caspase 3-like (*left*) and caspase 1-like (*right*) activities of BA/F3 cell lysate were analyzed by fluorescence-conjugated peptide substrates. **C**, Degradation of α -fodrin after depletion of mIL-3 in BA/F3 cells. The α -fodrin of the BA/F3 total cell lysate was analyzed by Western blotting. Arrowheads indicate α -fodrin; molecular sizes are shown in kilodaltons.

conjugated annexin V⁴⁰ and propidium iodide double staining and obtained essentially the same results (data not shown). The ICE/CED-3 family of cysteine proteases, now renamed caspases,⁴¹ play key roles in apoptosis.⁴²⁻⁴⁴ To examine whether the factor depletion aug-

ments the enzymatic activation of caspases in BA/F3 cells, we analyzed caspase 1-like³⁷ and caspase 3-like⁴⁴ enzyme activities by cleavage of fluorogenic peptide substrates specific for these enzymes with the use of a spectrofluorophotometer. BA/F-wild cells were cultured in

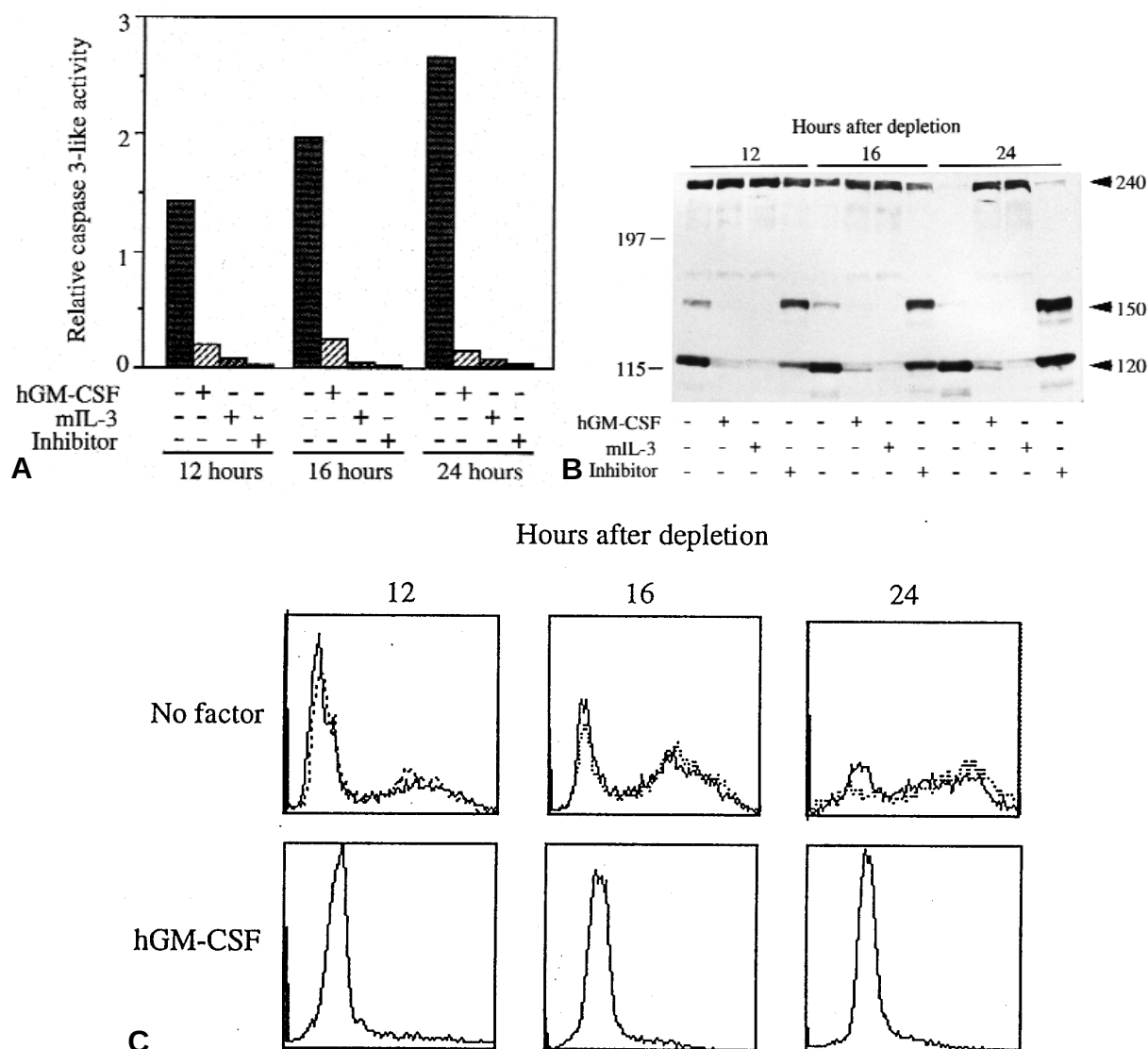


FIG 2. The effects of the inhibitor of caspase 3-like enzyme on factor depletion-induced apoptosis. The BA/F-3 wild cells were depleted of mIL-3 in the presence or absence of caspase 3-like enzyme inhibitor, for the indicated time. As control samples, cells were cultured in mIL-3 or hGM-CSF supplied medium; caspase 3-like enzyme activity (**A**), degradation of α -fodrin (**B**), and DNA fragmentation (**C**) were analyzed. In **C**, the *solid line* indicates samples without the inhibitor, and the *dotted line* indicates patterns obtained with the samples in the presence of inhibitor. Values are the average of 2 independent samples, and experiments, which were performed at least 3 times, gave essentially the same results.

the depletion medium, then enzyme activity of cell extract was determined at indicated time points. When we used caspase 3-specific substrate Ac-DEVD-MCA, augmentation of enzymatic activity was observed after 5 hours of factor depletion; the activity continuously increased for up to 24 hours after depletion (Fig 1, B). In contrast, when we used Ac-YVAD-MCA, a caspase 1-specific substrate,³⁷ no enzymatic activation was detected (Fig 1, B). We confirmed these results using another type of enzyme substrate, MocAc-YVADA PK(Dnp)-NH₂ and MocAc-DEVDA PK(Dnp)-NH₂,³⁸ and obtained essentially the same results (data not shown). Among several putative caspases, cellular substrate pro-

teins (α -fodrin is 1 of these) were reported to be degraded by caspase 3 from 240-kD into 150-kD and 120-kD fragments.⁴⁵ We next examined degradation of α -fodrin after factor depletion in BA/F3 cells by Western blotting of the total cell lysate. As shown in Fig 1, C, α -fodrin was initially degraded into 150 kD, and only the 120-kD fragment was observed after 24 hours of depletion.

Effects of caspase 3 inhibitor to depletion-induced apoptosis in BA/F3 cells

To investigate the role of the activation of caspase 3-like enzyme in apoptosis of BA/F3 cells, we analyzed the effects of peptide competitive inhibitor of caspase 3

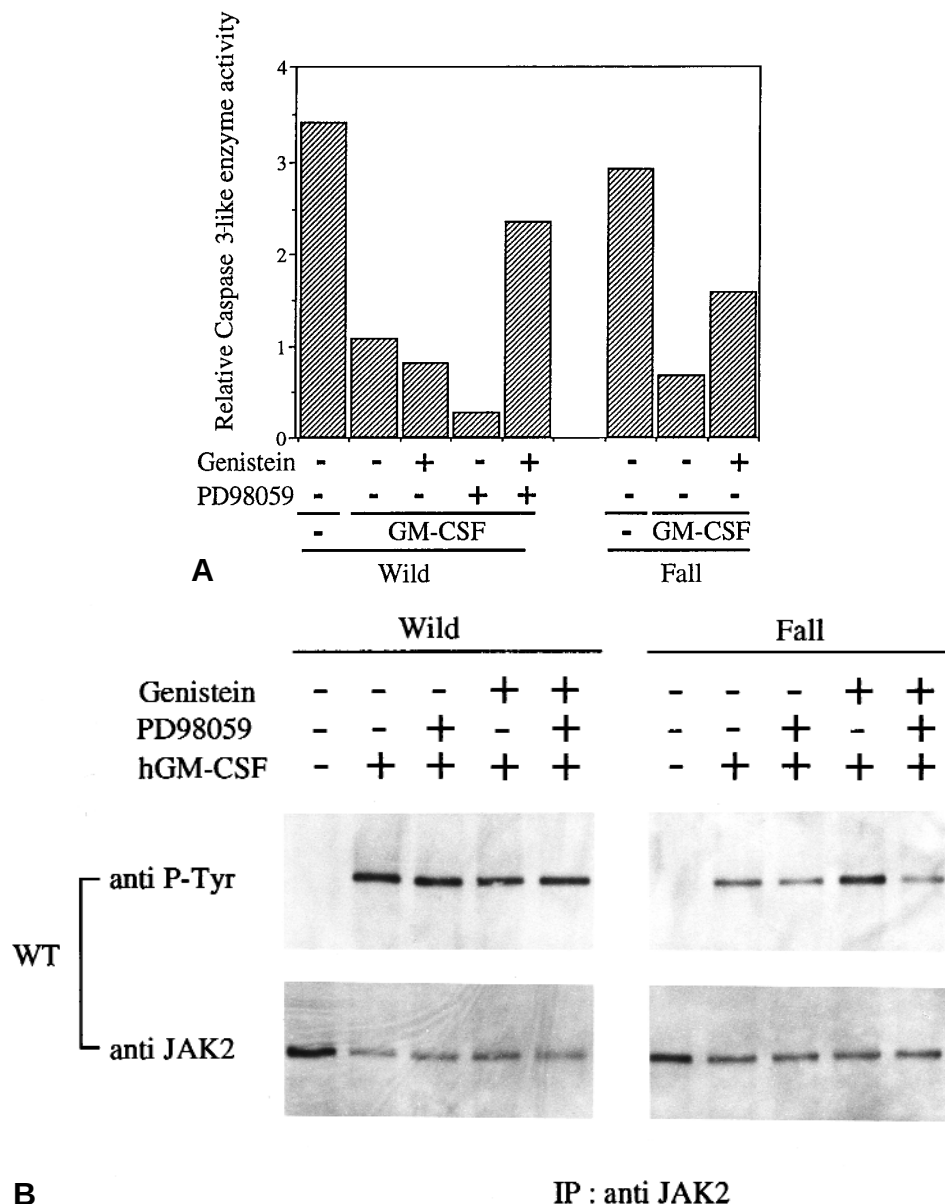


FIG 3. The effects of inhibitors on caspase 3 activity and JAK2 activity. **A**, Effect of PD98059 and genistein on depletion-induced caspase 3 activity. BA/F-wild cells were depleted of mIL-3 and restimulated with hGM-CSF in the presence of PD98059 and/or genistein. Caspase 3-like enzyme activity was analyzed after 24 hours of culture. The values are the average of 2 samples, and essentially the same results were obtained by 3 independent experiments. **B**, The effect of PD98059 and genistein for hGM-CSF induced JAK2 activity in BA/F3 cells. BA/F-wild cells were depleted of mIL-3 and restimulated with hGM-CSF for 10 minutes, and JAK2 was immunoprecipitated. Western blotting was performed with an antiphosphotyrosine antibody (4G10) or an anti-JAK2 antibody.

on factor depletion-induced apoptosis in BA/F3 cells. As shown in Fig 2, A, after 12 hours of depletion, a caspase 3-like enzyme is activated, as expected, and the addition of 400 $\mu\text{mol/L}$ of a peptide inhibitor A-Asp-Glu-Val-Asp-CH₂F completely suppressed this activity. A longer depletion period resulted in further activation of the enzyme, but no detectable activity was observed in the presence of the inhibitor. Although there was no evidence of caspase 3-like enzyme activity, α -fodrin degradation

was only partially suppressed by the inhibitor. As shown in Fig 2, B, in the absence of the inhibitor, complete degradation of α -fodrin to a predominantly 120-kd fragment was observed. In the presence of the peptide inhibitor, degradation was also observed, but the 150-kd fragment was predominant. Further factor depletion led to complete degradation of α -fodrin to the 120-kd fragment, although α -fodrin was mostly degraded to the 150-kd fragment in the presence of the inhibitor. These results

suggest that caspase 3–like enzyme activity is not essential for α -fodrin degradation, but delayed degradation suggested the involvement of caspase 3 or another enzyme with a similar substrate specificity to caspase 3. We next analyzed DNA fragmentation by TUNEL analysis in the presence or absence of the inhibitor. Interestingly, DNA fragmentation was not affected by the inhibitor at any time examined (Fig 2, C). It thus appears that caspase 3–like activity is not essential to promote DNA fragmentation in this system.

Effects of various inhibitors for antiapoptosis activity of hGM-CSF

We reported that there are at least 2 distinct signaling pathways of the hGMR, one for activation of the *c-myc* gene and cell proliferation and the other for activation of the *c-fos* gene.¹¹ These 2 pathways have different receptor domain requirements and different sensitivities to the tyrosine kinase inhibitor genistein.¹¹ Genistein inhibits GM-CSF-induced *c-myc* transcription and cell proliferation but does not affect the MAPK cascade,¹¹ which suggests that this tyrosine kinase inhibitor is an appropriate tool for examining these 2 signaling pathways. GM-CSF activates ERK and JNK, requiring the same β c tyrosine residues in both cases.^{16,46} MEK1 is an upstream activator of ERK, but not JNK, and PD98059 is an MEK1-specific inhibitor.^{47,48} We examined the effects of these inhibitors on caspase 3–like enzyme activation in BA/F-wild and BA/F-Fall cells (Fig 3, A). The addition of either PD98059 or genistein had no effect for anti-caspase 3–like enzyme activation, but only when both drugs were added, hGM-CSF lost this activity in BA/F-wild cells. In the case of BA/F-Fall, cells only possess the genistein sensitive pathway but not the PD98059 pathway; the addition of genistein significantly suppressed hGM-CSF-dependent anti-caspase 3–like enzyme activation. These results are essentially the same as those obtained by TUNEL analysis. Therefore, the genistein-sensitive signaling pathway appears to be sufficient to transduce viable signals induced by hGM-CSF in the mutant receptor Fall. With the wild-type receptor, it is suggested that either of these pathways is sufficient for hGM-CSF-dependent antiapoptosis. We next examined the effects of genistein and PD98059 on hGM-CSF-induced JAK2 phosphorylation. As shown in Fig 3, B, JAK2 phosphorylation by hGM-CSF through wild-type or Fall receptors was not affected, even in the presence of both inhibitors, which suggests that a molecule downstream of JAK2 may be targeted by these inhibitors.

JAK2 is necessary for antiapoptosis activity of hGM-CSF

We and other groups showed that JAK2 plays an essential role for various hGM-CSF activities and that JAK2 activation depends on the box1 region of β c.^{8,9} Because the deletion of the box1 region abrogated the ability of hGMR to maintain cell survival, the JAK2 seemed likely to play an essential role in transducing signals for antiapoptosis. To test this idea, we examined the

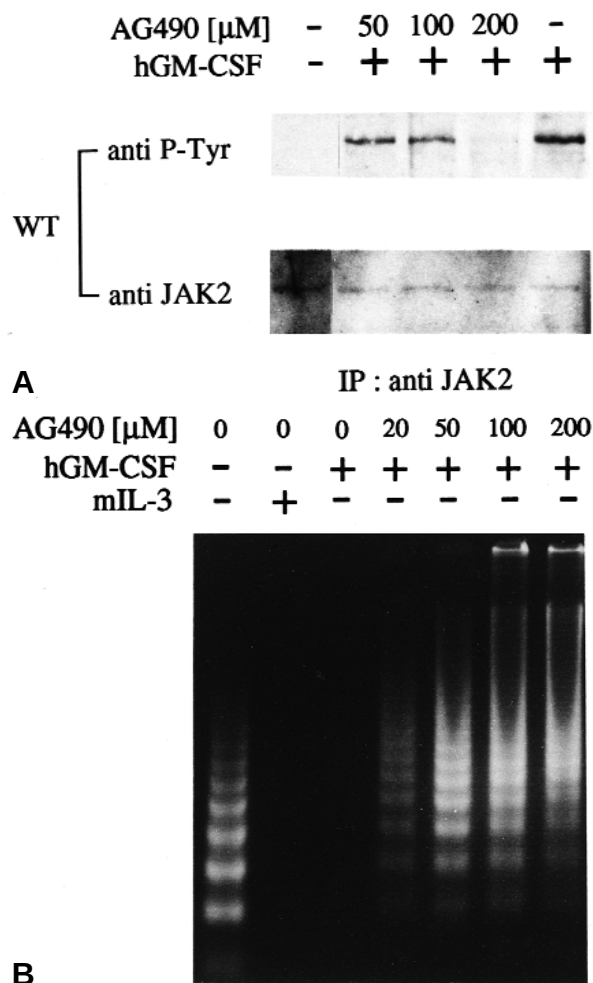


FIG 4. Effects of JAK2 inhibitor AG490 for the prevention of DNA fragmentation by hGM-CSF. **A**, The effects of AG490 on phosphorylation of JAK2 induced by hGM-CSF. BA/F-wild cells were stimulated for 10 minutes with hGM-CSF in the presence or absence of AG490, and immunoprecipitation (IP) of JAK2 followed by Western blotting was performed. For the blots, antiphosphotyrosine antibody 4G10 or anti-JAK2 antibody (Upstate Biotechnology, Incorporated) was used. WT, Western blotting. **B**, The effects of AG490 on anti-DNA fragmentation of hGM-CSF. DNA fragmentation was analyzed by gel electrophoresis.

effects of AG490, which is reported to be a specific inhibitor of JAK2,^{49,50} for anti-DNA fragmentation activity of hGM-CSF. We first examined the effects of AG490 for JAK2 phosphorylation that was induced by hGM-CSF by immunoprecipitation followed by Western blotting with an antiphosphotyrosine antibody. BA/F-wild was depleted of mIL-3 for 5 hours, and different doses of AG490 were added for the last 2 hours of the incubation, then the cells were restimulated with hGM-CSF (10 ng/mL) for 10 minutes. As shown in Fig 4, A, the addition of AG490 dose-dependently suppressed JAK2 phosphorylation, and phosphorylation of JAK2 was completely inhibited with up to 200 μ mol/L of AG490. We next examined the effect of AG490 on DNA fragmentation by TUNEL analysis. In this experiment,

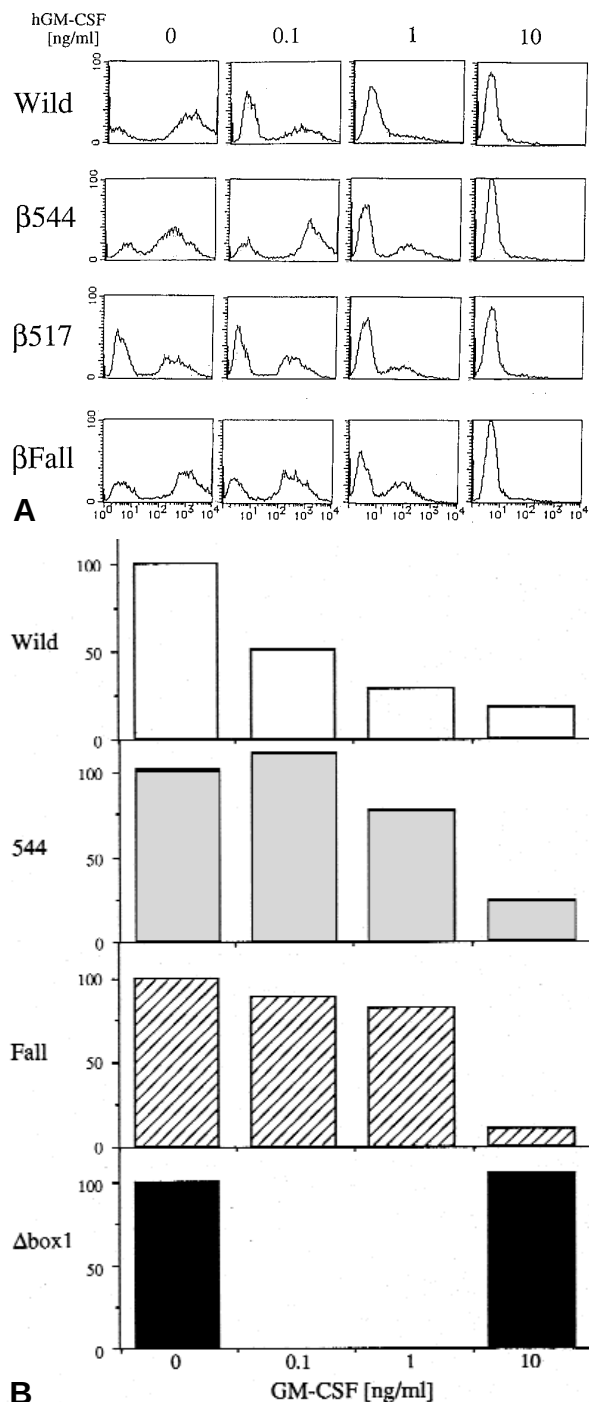


FIG 5. The effect of dose of hGM-CSF for antiapoptosis activity through various hGMR mutants in BA/F3 cells. BA/F-wild, -544, -517, and -Fall cells were cultured in different doses of hGM-CSF for 24 hours; DNA fragmentation (A) or caspase 3 enzymatic activity (B) was analyzed by TUNEL assay. The values are the average of 2 independent experiments.

although the cells were considered to be morphological nonviable, no fluorescence positive fraction was detected by flow cytometer. Because this phenomenon could be caused by the inhibition of enzymatic labeling

of fragmented DNA by AG490, we changed the protocol and examined DNA fragmentation using gel electrophoresis. BA/F-wild cells were preincubated with AG490 for 2 hours without growth factors, followed by incubation with hGM-CSF (10 ng/mL) for 22 hours. As shown in Fig 4, B, DNA fragmentation was not observed even in the presence of hGM-CSF in an AG490 dose-dependent manner. AG490 inhibited hGM-CSF-dependent prevention of the DNA fragmentation induced by factor depletion, which suggests that JAK2 has an essential role in mediating the antiapoptotic activity of hGM-CSF in BA/F3 cells.

The effects of hGM-CSF on antiapoptosis activity of wild and mutant hGMR

We next analyzed the effects of various doses of hGM-CSF for antiapoptosis activity of wild and mutant hGMR (544, 517, and Fall) expressed in BA/F3 cells. BA/F-wild, -544, -517, and -Fall cells were depleted and recultured with different doses of hGM-CSF for 24 hours, and DNA fragmentation was examined by the TUNEL assay (Fig 5, A). BA/F-wild cells survived perfectly in the presence of 1 or 10 ng/mL of hGM-CSF, but DNA fragmentation occurred within 0.1 ng/mL of hGM-CSF. However, the population of viable cells was lower than that seen without hGM-CSF, which suggests that 0.1 ng/mL of hGM-CSF still had some effect to prevent DNA fragmentation. In contrast, BA/F-544, -517, and -Fall cells could remain viable with 10 ng/mL of hGM-CSF, but a significant population of apoptotic cells appeared within 1 ng/mL of hGM-CSF. These cells cannot survive within 0.1 ng/mL of hGM-CSF, and the pattern of TUNEL analysis is almost the same as that observed within the absence of hGM-CSF. We next analyzed the activity of caspase 3 under the same condition. As shown in Fig 5, B, caspase 3 activity of BA/F-wild cells is suppressed in the presence of hGM-CSF dose-dependent manner. In the BA/F-544 and -517 cells, 10 ng/mL of hGM-CSF suppressed caspase 3 activity, but less than 1 ng/mL of hGM-CSF did not have an effect on the suppression. In the BA/F-Δbox1 cells, which cannot survive in hGM-CSF at all, activity of caspase 3 was induced in the presence of 10 ng/mL of hGM-CSF, as expected.

We found that the binding affinity of wild-type and Fall to hGM-CSF was equivalent in a binding assay with the use of iodide-labeled hGM-CSF (data not shown).

DISCUSSION

Using various hGMR βc mutants, we showed that only the box1 region of the βc is required to resist apoptosis that was induced by factor depletion or by γ irradiation in BA/F3 cells. Previous analyses that examined the activation of various signaling events by βc mutants indicated that the box1 region is essential for the activation of all the tested GM-CSF-induced phenomena.¹⁰ Effect of dominant negative JAK2 on GM-CSF signal transduction shows a similar phenotype to that observed with a Δbox1 mutant, βc, which lacks the box1 region.⁹ The binding of

overexpressed JAK2 to the membrane proximal region of β c containing box1 was noted in insect cells.⁸ Taken together, the essential role of JAK2 for all known GM-CSF-induced activities through the β c box1 region seems clear. Our results that used receptor mutants indicate a role for JAK2 in the antiapoptosis activity of GM-CSF. In the present study, more direct results that support the idea were obtained with the use of the JAK2 inhibitor, AG490. Consistent with our observations, recent works that used fusion protein of kinase domain of JAK2 and the extracellular domain of CD16 showed that artificial activation of JAK2 rescues BA/F3 cells from apoptosis and results in the increased expression of *bcl-2* gene and the *pim-1* gene.⁵¹ In addition, we examined the chimeric β c/JAK2 in BA/F3 cells⁵² and found that the BA/F- β /JAK2 survives in the absence of mIL-3. Thus, it can be speculated that depletion-induced apoptosis is completely prevented by signals from β /JAK2.

There are controversial results to examine whether MAPK cascade is required for antiapoptosis. We initially noted that the region that covers 46 amino acids between amino acids 544-589 is critical and sufficient for *c-fos* activation.¹⁰ Then, by constructing the Y series, we obtained various mutants (such as Y12, Y6, Y7 and Y8), which can promote proliferation without activation of MAPK cascade and *c-fos* activation.¹⁶ Further analyses with various β c mutants (including C terminal deletion and Y series mutants) revealed that, as long as we used 10 ng/mL of hGM-CSF, BA/F3 cells survive through mutants, 544, Y12, Y6, Y7 and Y8, lacking region or tyrosine residues required for MAPK cascade activation. That means our results are consistent through all the mutants. Interestingly, although the addition of 1 ng/mL of hGM-CSF was sufficient to maintain survival of BA/F-wild cells, BA/F-Fall cells could not survive, and DNA fragmentation occurred with this concentration of hGM-CSF. The affinity of Fall to hGM-CSF is almost the same as that of the wild-type receptor, and the level of receptor expression was similar in the clones used in this work.¹⁶ It is conceivable that the different biologic outcome may be due to differences in the number of firing receptors under these conditions. Although most of the current work on signal transduction has examined the "on/off" switching of each signaling event activated by growth factor, it should be noted that signaling firing may not be classified into simple "on" and "off" states in the native cells. Within the activation of wild type hGMR, all the signaling pathways (including MAPK cascade) may be activated; and in such cases, weak activation of all the events may be enough for antiapoptosis. On the other hand, in the case of Fall, which lacks the ability to activate MAPK and activates only genistein-sensitive pathway, full activation of this pathway is required for antiapoptosis. Consistent with the notion, other mutants, β 517 and 544, lacking MAPK cascade activation showed similar phenotype with that observed with Fall. Thus, our results support a hypothesis that, not only combining various signaling molecules but also balancing the strength of such events, may determine physiologic response.

Based on our series of experiments with various mutants hGMR and drugs, we can classify β c mutants: the first group activates proliferation, survival, and the MAPK cascade; the second group activates proliferation and survival but not the MAPK cascade. This biologic phenotype was observed in the presence of an excessive amount of hGM-CSF, and a low dose of hGM-CSF revealed more complex biologic response to the stimulation of receptors. Further studies may shed more light on the nature of signaling and biologic responses on the native cells and tissues.

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