INTRODUCTION

MM is a human B-cell neoplasia that is characterized by clonal expansion of malignant plasma cells that secrete monoclonal immunoglobulin. Myeloma cells are mainly localized in the bone marrow. Their proliferation, differentiation, and function are regulated by a complex network of cytokines and cell adhesion molecules, which are produced by bone marrow stromal cells. IL-6 supports the proliferation of myeloma cells in vitro and in vivo (1, 2), and certain myeloma cell lines respond to oncostatin M, IL-11, and leukemia inhibitory factor. Therefore, both IL-6 and IFN-α blocked DEX plus oIGF-1R-induced apoptosis through activation of the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways.

Cytokines Prevent Dexamethasone-induced Apoptosis via the Activation of Mitogen-activated Protein Kinase and Phosphatidylinositol 3-Kinase Pathways in a New Multiple Myeloma Cell Line

Megumu Ogawa, Tetsuo Nishiura, Kenji Oritani, Hitoshi Yoshida, Masafumi Yoshimura, Yu Okajima, Jun Ishikawa, Koji Hashimoto, Itaru Matsumura, Yoshiaki Tomiyama, and Yuji Matsuzawa

Department of Internal Medicine and Molecular Science, Graduate School of Medicine B5 [M. O., T. N., K. O., H. Y. M., Y. O., J. I., K. H., Y. T., Y. M.], and Department of Hematology and Oncology [I. M., Osaka University, Suita, Osaka 565-0871, Japan

ABSTRACT

A new human myeloma cell line, OPM-6, was established from the peripheral blood of a patient with advanced IgG-κ plasma cell leukemia. Cytogenetic and phenotypic analysis confirmed that the cells were derived from the patient’s leukemic cells. Insulin-like growth factor-1 (IGF-1) acts as an autocrine growth factor in these cells. In addition, OPM-6 cells were particularly sensitive to dexamethasone (DEX), when endogenous IGF-1 was blocked. Under these conditions, >95% of the DEX-treated cells died within 36 h. Therefore, OPM-6 represents a potentially powerful tool for the analysis of the molecular mechanisms of DEX-induced apoptosis, because it is possible to easily analyze the direct effects of DEX using this system. Using this culture system of OPM-6, we demonstrated that the treatment with DEX plus a monoclonal antibody to the human IGF-1 receptor (αIGF-1R) leads to the down-regulation of the gene expression of Bcl-xL, an antiapoptotic gene, and the activation of CPP32 during this apoptotic process. IFN-α as well as IL-6 prevented DEX plus oIGF-1R-induced apoptosis, and this prevention was blocked by the mitogen-activated protein kinase kinase inhibitor, PD098059, or the phosphatidylinositol 3-kinase inhibitor, wortmannin. Therefore, both IL-6 and IFN-α blocked DEX plus oIGF-1R-induced apoptosis through activation of the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways.

Received 10/12/99; accepted 5/24/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Japan Society for the Promotion of Science, and Senri Foundation.

2 To whom requests for reprints should be addressed, at Department of Internal Medicine and Molecular Science, Graduate School of Medicine B5, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan.

3 The abbreviations used are: MM, multiple myeloma; IL, interleukin; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; DEX, dexamethasone; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI3-K, phosphatidylinositol 3-kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

MATERIALS AND METHODS

A Case Report and Establishment of Cell Line. A 71-year-old female patient had bone fractures, elevation of a monoclonal IgG-κ (3.5 g/dl) in serum, and an accumulation of plasma cells (37.6%) in the bone marrow and was, therefore, diagnosed with MM. Despite treatment with IFN-α or two cycles of melphalan plus prednisolone for 1 year, she showed indication of plasma cell leukemia. Her peripheral blood contained a number of plasma cells

4262

Downloaded from cancerres.aacrjournals.org on January 5, 2018. © 2000 American Association for Cancer Research.
(34%), and her bone marrow aspirate revealed 83.0% of plasma cells at that time.

A new myeloma cell line, OPM-6, was established from her peripheral blood at the stage of plasma cell leukemia. Informed consent was given before obtaining samples of peripheral blood. The mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (Nycoderm Pharma AS, Oslo, Norway) density gradient. The mononuclear cells were washed three times in RPMI 1640 (Osaka Biken, Osaka, Japan) and resuspended in RPMI 1640 supplemented with 10% FCS (Flow, North Ryde, Australia) and cultured in humidified atmosphere with 5% CO2 at 37°C. No growth factors or feeder cells were used. The culture was maintained by partial replacement of spent medium with fresh medium (RPMI 1640 containing 10% FCS) at 3–4-day intervals. After 3 months, continuous growth of the cells was observed. We cloned these cells by two rounds of limiting dilution and established the OPM-6 cell line.

**Cells, Reagents, and Antibodies.** A human histiocytic lymphoma cell line, U937, was maintained in RPMI 1640 supplemented with 10% FCS. Recombinant human IL-6 was provided by Kirin Brewery Company Ltd. (Tokyo, Japan); recombinant human IFN-α was provided by Yamamoto Pharmaceutical Company Ltd. (Osaka, Japan); and recombinant human IFN-γ was provided by Yamanouchi Pharmaceutical Company Ltd. (Tokyo, Japan). DEX was purchased from Sigma Chemical Co. (St. Louis, MO). A murine anti-phosphotyrosine antibody, 4G10, was supplied by Dr. B. Druker (Oregon Health Science University, Portland, OR). A blocking antibody to the human IGF-1R (eChIP-IR) was purchased from Oncogene Research Products (Cambridge, MA), and its isotype-matched control antibody with irrelevant specificity, MOPC, was purchased from Sigma. A specific MAPK kinase (MEK) inhibitor (PD98059) and antibodies against the phosphorylated forms of p42/p44 MAPK were purchased from New England Biolabs (Beverly, MA); antibodies against pan-p42/p44 MAPK were from Zymed (San Francisco, CA); the PI3-K inhibitor (wortmannin) was from Sigma; and antibodies against caspase-3 (CPP 32) were from Santa Cruz Biotechnology (Santa Cruz, CA). Biotin-conjugated anti-Annexin V-antibody was a kind gift of Dr. Sakata (Osaka University, Osaka, Japan).

**Intracellular Immunoglobulin.** Cytoplasmic immunoglobulin was determined by direct immunohistochemistry as reported previously (15). Cytocentrifuged smears of cells were prepared, air-dried, and fixed in 70% ethanol. The slides were then incubated with 0.3% H2O2-methanol for 30 min to block myeloperoxidase reaction. After being washed in PBS, the cells were incubated with peroxidase-labeled anti-κ or anti-λ (DAKO Immunoglobulins, Copenhagen, Denmark) for 2 h. After washing in PBS, the conjugates were visualized a 4-chloro-1-naphthol-H2O2 reaction. Nuclei were counterstained with Mayer’s hematoxylin.

**Flow Cytometry Analysis.** Cells were incubated with the indicated antibodies at 4°C for 30 min, rinsed, and developed with FITC-conjugated goat antirabbit immunoglobulin (Becton Dickinson, Mountain View, CA) at 4°C for 30 min. The cells were rinsed and analyzed by FACSort (Becton Dickinson). Antibodies used in this study were as follows: mouse antihuman κ and λ were purchased from Becton Dickinson; mouse antihuman CD19 and CD38 were from Coulter (Lealeshe, FL); a monoclonal antibody to the human IGF-1 receptor (eChIP-1R) was from Oncogene Research Products.

**[3H]Thymidine Incorporation Assays.** To quantify the DNA synthesis of cells, we used a [3H]thymidine incorporation assay as described previously (16). Cells were washed three times with RPMI 1640 and seeded at 2.5 × 10^5 well in flat-bottomed 96-well plates in 0.2 ml of COS 004 medium, which is Copenhagen, Denmark) for 2 h. After washing in PBS, cells were fixed in 4% paraformaldehyde in PBS for 30 min and then transferred to a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After washing in PBS, the cells were exposed to the TUNEL reaction mixture, which contained the terminal deoxynucleotidyl transferase enzyme and fluorescein-dUTP, according to the protocol provided by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Incorporation of nucleotides into the 3′-DNA fragmented ends was then detected by flow cytometry. Apoptotic cells were also detected using annexin-V staining. Cells were washed twice in RPMI 1640 and resuspended in 100 μl of labeling solution containing 2 μl of biotin-annexin-V in PBS for 30 min at 4°C. The cells were then rinsed and developed with fluorescein-conjugated avidin (Becton Dickinson) at 4°C for 30 min. The stained cells were analyzed by flow cytometry.

**Northern Blot Analysis.** Total RNAs were isolated using the TRI-ol reagent (Life Technologies, Inc., Grand Island, NY), electrophoresed through formaldehyde-agarose gels, and transferred onto nylon membranes (Amer- sham). DNA fragments were labeled with [32P]dCTP using a random-primer DNA labeling kit (Boehringer Mannheim) and hybridized to the membranes. Blots were then washed and autoradiographed. The CDNA fragments of IGF-1, Bcl-2, Bcl-xl, Bak, p53, and β-actin were used as materials for probes. Blots were scanned and quantitatively analyzed by NIH Image.

**Western Blot Analysis.** The isolation of cellular lysates, immunoprecipitation, gel electrophoresis, and immunoblotting were performed according to methods described previously (17). The cells, which were stimulated with the indicated factors, were lysed in lysis buffer containing 0.5% Triton X-100, 50 mM HEPES (pH 7.5), 100 mM NaF, 10 mM sodium phosphate, 4 mM EDTA, 2 mM NaVO4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml phenyl- methylsulfonyl fluoride, and the resulting insoluble material was removed by centrifugation. The protein concentration of each sample was estimated using the Pierce Micro BCA protein assay kit (Pierce, Rockford, IL). The cell extracts (18–30 μg of protein) were electrophoresed in 10% (w/v; for MAPK detection) or 16% (w/v; for CPP32 detection) SDS-polyacrylamide gel in a reduced condition, and were then electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA). The membranes were blocked overnight in TBST (20 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) containing 2.5% BSA and then incubated for 2 h with the appropriate primary antibody in TBST. After washing, the membranes were incubated with appropriate horseradish peroxidase-coupled secondary antibodies (Promega Corp., Madison, WI) for 1 h before detection of signals by enhanced chemiluminescence detection system (DuPont NEN, Boston, MA).

**PI3-K Activity Assay.** After stimulation, the cells were solubilized in lysis buffer (10 μM HEPES (pH 7.5), 0.15 mM NaCl, 1% NP40, 10% glycerol, 10 μg/ml each leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na2VO4, and 5 mM EDTA) (18). Nuclei and debris were removed by centrifugation, and the proteins were subjected to immunoprecipitation for 2 h at 4°C with the anti-phosphotyrosine antibody, 4G10. Immunocomplexes were collected with protein G-Sepharose that had been preconjugated with a rabbit antiserum IgG antibody and sequentially washed twice in lysis buffer, twice in LiCl buffer (HEPES buffer containing 0.5 mM LiCl and 0.2% NP40), and finally, in 10 mM HEPES and 0.15 mM NaCl. Then, 30 μl of 10 mM phenyl phosphatase were added, and the eluate was used for the PI3-K assay. The immunocomplexes were incubated with 0.2 mg/ml i-γ-phosphatidilinositol, 40 mM ATP, 30 mM MgCl2, and 20 μCi of [γ-32P]ATP for 10 min at 37°C. The reactions were terminated with 200 μl of 1 N HCl, and lipids were extracted with 200 μl of chloroform:methanol (1:1, v/v). After washing with methanol/1 N HCl (1:1, v/v), phosphorylated lipids were then extracted and resolved by TLC using chloroform:methanol:H2O:NH4OH (43:38:7.5, v/v) as solvent. Radioactive spots were detected by autoradiography.

**Statistical Analysis.** Data were analyzed by the Student’s t tests, and results were expressed as mean ± SD.

**RESULTS**

**Characterization of a Newly Established Myeloma Cell Line, OPM-6.** A new myeloma cell line was established from the peripheral blood cells of a patient with plasma cell leukemia. Persistent cell proliferation was observed after 3 months of incubation. The cell line was maintained by adding fresh medium at 3–4-day intervals. At the time of writing, the cell line was 50 months old and has been...
designated OPM-6. OPM-6 cells were free from Epstein-Barr virus, grew as a suspension of single cells, and did not attach to the dish (data not shown). OPM-6 cells showed plasmacytoid features, which were characterized by round nuclei and large basophilic cytoplasm (Fig. 1A). OPM-6 expressed CD38, a typical marker for plasma cells, but not CD19, a typical marker for pan B cells (Fig. 1B). Moreover, immunohistochemical analysis has shown that the intracellular IgG-k chain was clearly present in the cytoplasm (Fig. 1C). JH alleles of OPM-6 were rearranged, and the rearranged bands were identical to those of the patient’s DNA by Southern blot (data not shown). These facts indicate that OPM-6 was established from leukemic cells of the patient.

Autoocrine IGF-1 Growth Loop in OPM-6 Cells. A number of growth factors affect the proliferation of myeloma cells and sometimes act as autocrine growth and survival factors. We observed that IGF-1 and IL-6 could stimulate OPM-6 DNA synthesis in the [3H]thymidine incorporation assays (Fig. 2A and data not shown). IFN-α failed to inhibit or rather slightly stimulated DNA synthesis in OPM-6 (data not shown). Similar stimulatory effects were observed in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Among these cytokines, OPM-6 expressed IGF-1 messages of three different sizes in Northern blot analysis (Fig. 2B). In contrast, the gene expressions of IL-6 and IFN-α were not detected by either Northern blot analysis or the reverse transcription-PCR (data not shown). In addition, OPM-6 cells expressed IGF-1 receptors on their surface in flow cytometry analysis (Fig. 2C). A blocking monoclonal antibody of the IGF-1 receptor (αIGF-1R) could inhibit up to 10 ng/ml exogenous IGF-1-stimulated DNA synthesis (data not shown). The addition of αIGF-1R also inhibited the spontaneous OPM-6 DNA synthesis in a dose-dependent manner (Fig. 2D), whereas it had no effect in U266 myeloma cell line (data not shown). The similar results were detected in the cases of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). These findings suggest that the autocrine IGF-1 loop may contribute to the proliferation of OPM-6 cells.

DEX Plus αIGF-1R Induces Apoptosis in OPM-6. DEX is used frequently in the treatment of patients with MM. We evaluated the influence of DEX on the proliferation of OPM-6 cells. DEX at concentrations of 10⁻² to 10⁻¹ M showed only minor cytotoxic effects on OPM-6 cells (Fig. 3A). Because IGF-1 acts as an autocrine growth factor in OPM-6 cells, we treated OPM-6 cells with DEX in the presence or absence of αIGF-1R. As shown in Fig. 3A, the viability of OPM-6 cells was influenced by DEX at a concentration of 10⁻¹ M, when IGF-1 receptors on OPM-6 cells were blocked by the antibody, and a maximal activity was obtained with 5 x 10⁻⁶ M DEX. After treatment with 5 x 10⁻⁶ M DEX plus αIGF-1R for 36 h, >95% of OPM-6 cells died (Fig. 3B).

Fig. 1. Characterization of OPM-6, a myeloma cell line. A, OPM-6 cells were prepared by cytopsin, stained by May-Grünwald-Giemsa, and photographed at ×1000. B, surface expressions of CD19 and CD38 on OPM-6 were evaluated by flow cytometry. C, OPM-6 cells were prepared by cytopsin, stained with anti-immunoglobulin light chain-specific antibodies, and photographed at ×400.

Fig. 2. Autocrine IGF-1 growth loop in OPM-6 cells. A, effect of IGF-1 on DNA synthesis of OPM-6. OPM-6 cells (2 x 10⁴/well) were cultured in 96-well plates in a serum-free medium with the indicated concentrations of IGF-1 for 24 h. The DNA synthesis was evaluated using a [3H]thymidine incorporation assay. Bars, SD. B, IGF-1 gene expression in OPM-6. Total RNAs (15 μg/lane) were isolated using TRI-zol reagent and subjected to Northern blot analysis with a probe for IGF-1 cDNA. Lower panel, a control with equal loading where the same blot was probed with β-actin. C, surface expression of IGF-1 receptor in OPM-6 cells. Cells were stained with αIGF-1R, a murine anti-human IGF-1 receptor antibody, followed by FITC-goat antimouse IgG (shaded histogram). Negative control staining, obtained with an isotype-matched control murine antibody, is also shown (open histogram). D, OPM-6 cells (2 x 10⁴/well) were cultured in 96-well plates in a serum-free medium with αIGF-1R (●) or MOPC (mouse IgG1; ■) for 24 h. The DNA synthesis was evaluated using a [3H]thymidine incorporation assay. The results are shown as the means of triplicate cultures; bars, SD.
Cytokines Prevent Dexamethasone-Induced Apoptosis

To investigate whether the DEX plus αIGF-1R-induced cell death was apoptosis, OPM-6 cells, treated either with DEX alone or DEX plus αIGF-1R, were analyzed by the TUNEL assay and Annexin-V staining. When OPM-6 cells were incubated with DEX alone for 24 h, ~10% of the cells were detected as apoptosis in the TUNEL assay (Fig. 3C, upper histogram). The addition of αIGF-1R dramatically exacerbated DEX-induced apoptosis, increasing it from 10 to 80% in OPM-6 cells (Fig. 3C, lower histogram). We next stained OPM-6 cells with Annexin-V, a reagent that detects phosphatidylserine present in the outer membrane of apoptotic cells (19). Similar results were obtained, i.e., ~80% of OPM-6 cells treated with DEX plus αIGF-1R were Annexin-V positive (Fig. 3D). The fact that DEX markedly induces apoptosis in OPM-6 cells in the presence of αIGF-1R.

DEX Modulates the Expression of Apoptosis-related Genes and the Activity of the Caspase Family. To investigate molecular mechanisms underlying the DEX plus αIGF-1R-induced apoptosis in OPM-6 cells, we evaluated the expression of some apoptosis-related genes by Northern blot analysis. As shown in Fig. 4A, OPM-6 cells constitutively expressed Bcl-xL, Bak, and p53 genes. Expression of Bak and p53 genes was not affected by DEX plus αIGF-1R, and the Bcl-2 gene was not expressed at a level that was detectable by Northern blot analysis. However, down-regulation of the Bcl-xL gene expression was observed in OPM-6 cells as early as 2 h after treatment with DEX plus αIGF-1R (Fig. 4). We further investigated the issue of whether treatment with DEX correlated with the activation of caspases, which is a major pathway of apoptosis (8). The activation of CPP32, caspase-3, was monitored by Western blot analysis, because its activation causes the cleavage of its M₁, 32,000 precursor into M₂, 17,000 and M₂, 12,000 fragments (8). CPP32 was activated within 16 h in the case of the DEX-treated OPM-6 cells and within 8 h in the DEX plus αIGF-1R-treated OPM-6 cells (Fig. 5). Therefore, DEX plus αIGF-1R modulates gene expression of Bcl-xL and activates CPP32 in OPM-6.

Antiapoptotic Signaling Pathways Downstream from the IL-6 and IFN-α Receptors. In several cell systems, cytokines have protective effects against apoptosis (10, 20). Thus, we examined the issue that treatment with DEX plus αIGF-1R down-regulates the gene expression of Bcl-xL. A, expression of apoptosis-related genes during culture with DEX plus αIGF-1R (upper panel). OPM-6 cells were cultured with 5 × 10⁻⁴ M DEX plus 1 μg/ml αIGF-1R for the indicated periods. Total RNAs (15 μg/lane) were isolated and subjected to Northern blot analysis using cDNAs of Bcl-2, Bcl-xL, Bak, p53, and β-actin as probes. B, OPM-6 cells were cultured with 5 × 10⁻⁴ M DEX plus 1 μg/ml αIGF-1R for the indicated periods. Total RNAs (15 μg/lane) were isolated and subjected to Northern blot analysis using cDNAs of Bcl-xL and β-actin as probes. The same RNA blot was quantified by densitometric scanning and normalized with respect to β-actin mRNA. The relative expression of Bcl-xL mRNA was calculated based on Bcl-xL mRNA levels detected in control cells at the start.
of whether IL-6 or IFN-α is able to influence DEX-induced apoptosis in OPM-6. Treatment with DEX plus αIGF-1R decreased the percentage of viable cells at 24 h from 78.9 ± 3.1% to 25.4 ± 3.1%. However, in the presence of IL-6 or IFN-α, the viability was 80.4 ± 1.5% and 78.5 ± 3.5%. This was confirmed by the fact that both IL-6 and IFN-α significantly reduced apoptotic cells, which were detected by the TUNEL assay in DEX plus αIGF-1R-treated OPM-6 cells (Fig. 6A; 87.7 ± 7.8% with DEX + αIGF-1R; 20.3 ± 5.1% with DEX + αIGF-1R + IL-6; 19.9 ± 7.7% with DEX + αIGF-1R + IFN-α). Similarly, both IL-6 and IFN-α inhibited activation of CPP32 induced by DEX plus αIGF-1R in OPM-6 cells (Fig. 6B). These data indicate that IL-6 and IFN-α prevent DEX plus αIGF-1R-induced apoptosis and CPP32 activation in OPM-6.

To clarify the molecular mechanisms involved in IL-6- or IFN-α-mediated prevention of DEX plus αIGF-1R-induced apoptosis, we analyzed signals that were activated by these factors. The activation of p42/p44 MAPK, extracellular signal-regulated kinase 1/2, by IL-6 or IFN-α was detected by Western blot; and PD98059, a specific inhibitor of the activation of MEK (21), effectively inhibited this activation (Fig. 7B). Stimulation of OPM-6 cells with IL-6 or IFN-α also induced a marked increase in PI3-K activity, and this activation was blocked by wortmannin, a specific inhibitor of PI3-K (Ref. 22; Fig. 7A).

Because both IL-6 and IFN-α stimulate the signals of the Ras-MAPK and PI3-K pathways, we examined the possible role of these signals in the IL-6- or IFN-α-mediated prevention of DEX-induced apoptosis. As above, apoptosis of OPM-6 cells was induced by treatment with DEX plus αIGF-1R, and IL-6 or IFN-α blocked this induction. In this culture system, the activation of MEK or PI3-K by IL-6 or IFN-α was specifically blocked by pretreatment with PD98059 or wortmannin, respectively. DEX plus αIGF-1R-induced apoptosis in the presence of these inhibitors was evaluated by the TUNEL assay. As shown in Fig. 8A, pretreatment of OPM-6 cells with PD98059 completely canceled the IL-6- and IFN-α-mediated prevention of DEX plus αIGF-1R-induced apoptosis (20.3 ± 5.1% with DEX + αIGF-1R + IL-6; 84.7 ± 12.7% with DEX + αIGF-1R + IL-6 + PD98059; 19.9 ± 7.7% with DEX + αIGF-1R + IFN-α; 77.6 ± 9.8% with DEX + αIGF-1R + IFN-α + PD98059). As shown in Fig. 8B, pretreatment of OPM-6 cells with wortmannin partially canceled the IL-6- and IFN-α-mediated prevention of DEX plus αIGF-1R-induced apoptosis (20.3 ± 5.1% with DEX + αIGF-1R + IL-6; 41.5 ± 4.7% with DEX + αIGF-1R + IL-6 + wortmannin; 19.9 ± 7.7% with DEX + αIGF-1R + IFN-α; 38.9 ± 13.3% with DEX + αIGF-1R + IFN-α + wortmannin). Therefore, the activation of both MAPK and PI3-K is necessary for antiapoptotic signaling by IL-6 and IFN-α in OPM-6 cells.

**DISCUSSION**

In this study, we established a novel human myeloma cell line, OPM-6, from the peripheral blood of a patient with IgG-κ plasma cell leukemia. OPM-6 cells show plasma cell features, because they express CD38 on their surface and an IgG-k chain in their cytoplasm. We have demonstrated that IGF-1 acts as an autocrine growth factor in OPM-6. The evidence for this is as follows: (a) IGF-1 induces the proliferation of OPM-6; (b) OPM-6 cells produce IGFl; (c) OPM-6 cells express IGFl receptors on their surface; and (d) an anti-IGFl receptor antibody markedly blocks the spontaneous DNA synthesis of OPM-6. OPM-6 cells are particularly sensitive to DEX in the presence of αIGF-1R. In addition, we demonstrated that both IL-6 and IFN-α blocked DEX plus αIGF-1R-induced apoptosis in OPM-6 cells and that the activation of the Ras-MAPK and PI3-K pathways were
involved in the prevention of DEX plus αIGF-1R-induced apoptosis by IL-6 or IFN-α.

A variety of growth factors promote the proliferation of myeloma cells in vitro and/or in vivo. IL-6 is the most important growth factor for myeloma cells (1, 2), and an autocrine IL-6 loop sometimes operates in myeloma cell lines (23, 24). It is known that IGF-1 acts as a paracrine growth factor for certain myeloma cell lines (25), and an autocrine IGF-1 loop may participate in maintaining growth and survival of some myeloma cell lines, such as LP-1, Karpas 707, and DP-6, as well as others (20, 26). In OPM-6 cells, both IL-6 and IGF-1 significantly stimulated DNA synthesis. In addition, we showed that the autocrine IGF-1 loop operated in OPM-6 cells. In Northern blot analysis, we found that OPM-6 cells expressed a fair amount of IGF-1 gene. In addition, normal plasma cells and some myeloma cell lines as well as OPM-6 express IGF-1Rs (26, 27). Collectively, these data indicate that IGF-1 may function as a possible autocrine growth factor of myeloma cells.

Glucocorticoids, including DEX, play a key role in rheumatoid arthritis, collagen disease, lymphocytic leukemias, lymphomas, and MM therapy. (28). One relevant mechanism may be the induction of cell death. DEX induces apoptosis in a variety of cells, and the possible mechanisms have been discussed (29, 30). DEX treatment of a leukemic cell line repressed c-myc oncogene expression (31, 32) and activator protein-1 activity (31, 33). The overexpression of Bcl-2 in lymphoid cell lines blocks glucocorticoid-induced apoptosis (34). DEX-induced apoptosis is also dependent on protein kinase A and protein kinase C activity (34, 35), as well as caspase activation (35, 36). However, the molecular mechanisms induced by DEX remain unclear. We established a culture system to analyze the molecular mechanisms of DEX-induced apoptosis. OPM-6 cells were serum starved in the presence of αIGF-1R, and then 5 × 10^-6 M DEX were added to the culture. Under these conditions, >90% of the treated cells died by apoptosis within 36 h. Therefore, it is possible to analyze direct effects of DEX easily, and OPM-6 represents a potentially powerful tool for the analysis of the molecular mechanisms of DEX-induced apoptosis. Using the present culture system for OPM-6 cells, we demonstrated that DEX plus αIGF-1R down-regulated gene expression of Bcl-xL, an antiapoptotic gene, as early as 2 h. However, DEX plus αIGF-1R had no influence on the expressions of Bak and p53. In addition, DEX plus αIGF-1R induced the activation of CPP32 within 8 h. Therefore, the down-regulation of Bcl-xL and the activation of CPP32 seem to be important events for DEX plus αIGF-1R-induced apoptosis in OPM-6. It is important to understand the nature of the signals that are related to the DEX plus αIGF-1R-induced activation of CPP32. Some proapoptotic genes, except for Bak and p53, may activate CPP32 in response to DEX. Further analysis will provide clear answers to the question.

Several growth factors support the survival of myeloma cells and can prevent apoptosis in myeloma cells. IL-6 and IFN-α are known to inhibit the DEX-induced apoptosis of myeloma cells (10, 11, 36). Chauhan et al. (36) reported that DEX-induced apoptosis is associated with down-regulation of MAPK and p70S6K, and that IL-6 prevents this down-regulation. Yao and Cooper et al. (37) and Minshall et al. (38) reported that IGF-1-activated PI3-K plays a role in protecting against apoptosis. Recently, IFN-α has also been reported to be a survival factor against apoptosis induced by Apo-1/Fas (CD95; Ref. 13) or DEX-induced apoptosis of some myeloma cell lines (39, 40). Although it has been reported that IFN-α increases activator protein-1 activity by the activation of signal transducer and activator of transcription proteins (40) or induces the activation of a protein kinase C pathway (13), the exact role is currently unresolved. In OPM-6, we...
showed that DEX plus αIGF-1R-induced apoptosis was blocked by IL-6 or IFN-α. Treatment with IL-6 or IFN-α also blocked DEX plus αIGF-1R-induced activation of CPP32. The addition of αIGF-1R to OPM-6 cells with DEX dramatically exacerbated apoptosis, suggesting that the endogenous IGF-1 protects DEX-induced apoptosis in OPM-6 cells. We also demonstrated that activation of the Ras-MAPK and PI3-K pathways were important in preventing DEX plus αIGF-1R-induced apoptosis in OPM-6 cells. The Western blot analysis and PI3-K assay showed that IL-6 and IFN-α significantly and specifically stimulated MAPK and PI3-K in OPM-6 cells. In particular, the activation of the Ras-MAPK pathway is essential for the prevention of DEX-induced apoptosis, because the addition of PD98059, a specific inhibitor of the activation of MEK, completely canceled the IL-6- and IFN-α-mediated prevention of DEX-induced apoptosis. Our observations support the result of Chauhan et al. (36). Thus, our observations may not be restricted to OPM-6 and may be general. In addition, this is the first report that the Ras-MAPK and the PI3-K pathways mediate the antiapoptotic effect of IFN-α.

It is known that IFN-α has growth-inhibitory effects on myeloma cells (41). One mechanism involves the down-regulation of IL-6 receptors by IFN-α, thereby inhibiting IL-6 dependent growth of myeloma cells (42, 43). Another involves the direct modulation of the cell cycle of myeloma cells by IFN-α (44). However, IFN-α stimulates the proliferation of fresh myeloma cells from 16 to 50% in patients (4). Moreover, despite encouraging results in an early clinical trial with adjunct IFN-α treatment after chemotherapy (45), more recent studies have failed to demonstrate a benefit for IFN-α maintenance (46–48). Our data, as well as those of Ferlin-Bezombes et al. (39) and Liu et al. (40), demonstrate that IFN-α can prevent DEX-induced apoptosis of myeloma cells in vitro (39, 40). Therefore, IFN-α may also be a myeloma cell survival factor in some patients. Such adverse effects may help to explain why IFN-α therapy has only a partial response in some patients with MM.

Some strategies to block the effects of IL-6 have been exploited therapeutically, because it plays a central role in the proliferation of myeloma cells. All-trans retinoic acid inhibits IL-6-dependent growth of myeloma cells, decreases IL-6 production by myeloma and bone marrow stromal cells, and down-regulates IL-6 receptor expression (49, 50). Glucocorticoids, including DEX, can repress IL-6 or IFN-α-mediated prevention of DEX-induced apoptosis, because the addition of PD098059, a specific inhibitor of the activation of mitogen-activated protein kinase kinase (MEK), completely canceled the IL-6 and IFN-α-mediated prevention of DEX-induced apoptosis. Our observations support the result of Chauhan et al. (36). Thus, our observations may not be restricted to OPM-6 and may be general. In addition, this is the first report that the Ras-MAPK and the PI3-K pathways mediate the antiapoptotic effect of IFN-α.

ACKNOWLEDGMENTS

We thank Dr. Odajima for excellent technical assistance with the PI3-K assay.

REFERENCES

6. Jourdan, M., Zhang, X. G., Zhang, D., Moreau, F., and Pouyssegur, J. Interferon α inhibits Jun D activation, which is another growth factor of myeloma cells. We also demonstrated that activation of the Ras-MAPK pathway is essential for preventing DEX-induced apoptosis, because the addition of PD098059, a specific inhibitor of the activation of MEK, completely canceled the IL-6- and IFN-α-mediated prevention of DEX-induced apoptosis. Our observations support the result of Chauhan et al. (36). Thus, our observations may not be restricted to OPM-6 and may be general. In addition, this is the first report that the Ras-MAPK and the PI3-K pathways mediate the antiapoptotic effect of IFN-α.

It is known that IFN-α has growth-inhibitory effects on myeloma cells (41). One mechanism involves the down-regulation of IL-6 receptors by IFN-α, thereby inhibiting IL-6 dependent growth of myeloma cells (42, 43). Another involves the direct modulation of the cell cycle of myeloma cells by IFN-α (44). However, IFN-α stimulates the proliferation of fresh myeloma cells from 16 to 50% in patients (4). Moreover, despite encouraging results in an early clinical trial with adjunct IFN-α treatment after chemotherapy (45), more recent studies have failed to demonstrate a benefit for IFN-α maintenance (46–48). Our data, as well as those of Ferlin-Bezombes et al. (39) and Liu et al. (40), demonstrate that IFN-α can prevent DEX-induced apoptosis of myeloma cells in vitro (39, 40). Therefore, IFN-α may also be a myeloma cell survival factor in some patients. Such adverse effects may help to explain why IFN-α therapy has only a partial response in some patients with MM.

Some strategies to block the effects of IL-6 have been exploited therapeutically, because it plays a central role in the proliferation of myeloma cells. All-trans retinoic acid inhibits IL-6-dependent growth of myeloma cells, decreases IL-6 production by myeloma and bone marrow stromal cells, and down-regulates IL-6 receptor expression (49, 50). Glucocorticoids, including DEX, can repress IL-6 gene transcription and production (14, 51). More specific antagonists of IL-6 dependent transcription and production (14, 51). More specific antagonists of IL-6 dependent growth are the anti-IL-6 monoclonal antibody or the anti-IL-6 receptor antibody. Both reagents have been shown to suppress the proliferation of myeloma cells in vitro and in vivo (52–54). Chemically synthesized peptides also block the interaction between the IL-6 receptor α chain and gp130 (55). Our data indicate that DEX plus αIGF-1R-induced apoptosis of myeloma cells is blocked by IL-6. Therefore, the combination therapies involving DEX and IL-6 antagonists may induce the apoptosis of myeloma cells more dramatically in vivo. In addition, similar strategies to IL-6 may be used for IGF-1, which is another growth factor of myeloma cells. We also demonstrated that the activation of the Ras-MAPK pathway is essential for antiapoptotic signaling by IL-6 or IFN-α. These findings indicate that suppression of the Ras-MAPK pathway may increase sensitivity to DEX in myeloma cells. Recently, the Ras-MAPK pathway can be blocked by antisense oligonucleotides (56). In addition, the recently discovered inhibitors of Ras farnesyltransferases block the action of Ras (57). It would be extremely interesting to investigate whether these antagonists of the Ras-MAPK pathway are capable of augmenting the effects of DEX in vivo.


Cytokines Prevent Dexamethasone-induced Apoptosis via the Activation of Mitogen-activated Protein Kinase and Phosphatidylinositol 3-Kinase Pathways in a New Multiple Myeloma Cell Line

Megumu Ogawa, Tetsuo Nishiura, Kenji Oritani, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/15/4262

Cited articles  This article cites 55 articles, 31 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/15/4262.full#ref-list-1

Citing articles  This article has been cited by 20 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/15/4262.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/60/15/4262.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.