Sensitive Inhibitory Effect of Interferon-Alpha on M-Protein Secretion of Human Myeloma Cells

By Hideo Tanaka, Osamu Tanabe, Koji Iwato, Hideki Asaoku, Hideaki Ishikawa, Masaharu Nobuyoshi, Michio Kawano, and Atsushi Kuramoto

The effects of interferon-alpha (IFNa) on in vitro proliferation and M-protein secretion in human myeloma cells were investigated. Human myeloma cells were purified from bone marrow aspirates in 12 multiple myeloma patients. Purified myeloma cells were cultured for 48 hours with IFNa at its lower concentrations (0.1 to 100 U/mL). The cells were then pulsed with \(^{3}H\)-TdR for the last 12 hours and \(^{3}H\)-TdR uptake was measured (in vitro proliferation). After 48-hour culture, supernatants were harvested and the amount of M-protein in these fluids was measured by enzyme-linked immunosorbent assay (ELISA) (in vitro protein secretion). In vitro M-protein secretions of these myeloma cells were significantly suppressed even at 0.1 U/mL of IFNa, while \(^{3}H\)-TdR uptakes were not so suppressed until 10 or 100 U/mL of IFNa were added. The expressions of secretory immunoglobulin (Ig) mRNA of these myeloma cells were also selectively suppressed by IFNa. Furthermore, after IFNa had been administered intramuscularly, 3 to 6 \(\times\) 10^4 U/d for at least 1 month, in vitro M-protein secretions of these myeloma cells were decreased compared with those before IFNa administration. Therefore, these results suggest that IFNa has more sensitive inhibitory effect on M-protein secretion of human myeloma cells rather than on myeloma cell proliferation.

© 1989 by Grune & Stratton, Inc.

From the Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Japan. Submitted December 12, 1988; accepted May 8, 1989. Supported in part by grants from the Ministry of Education Science and Culture. Address reprint requests to Michio Kawano, MD, Myeloma Study Group, Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Kasumi-i-2-3, Minami-ku, Hiroshima 734, Japan.

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

© 1989 by Grune & Stratton, Inc.

0004-4971(89)70405-0 131$00/0

ANTITUMOR ACTIVITY of natural leukocyte interferon-α (IFNa) in patients with multiple myeloma has been reported in early clinical studies. Subsequent studies with lymphoblastoid, fibroblastoid, and recombinant interferons have confirmed their antitumor activities against multiple myeloma. Clinical trials are still under way to evaluate the efficacy of interferons in treatment of multiple myeloma. The rate of clinical response (a more than 50% decrease in serum M-protein and/or urine Bence-Jones protein levels) with various dose schedules and different IFNa was reported 8% to 33%. But the precise mechanism of the antitumor activity on multiple myeloma is, at present, still unclear. In this study, by using highly purified fresh myeloma cells obtained from myeloma patients, we have examined the effects of IFNa on in vitro proliferation, M-protein secretion, and expression of immunoglobulin (Ig) mRNA in myeloma cells. Furthermore, when IFNa was actually administered to myeloma patients, the effect of IFNa on in vitro M-protein secretion activity on myeloma cells was also examined.

MATERIALS AND METHODS

Patients.Bone marrow aspirates were obtained from 12 patients of multiple myeloma, ten IgG type and two IgA, before and after undergoing treatment. Prior chemotherapy was interrupted at least 1 month before sampling the bone marrow cells. Their age ranged from 51 to 76 (mean, 66.7); there were six men and six women. Clinical staging in these patients was performed according to the classification of Durie and Salmon. One patient was stage I, three patients stage II, and eight patients stage III. Informed consent was obtained before bone marrow aspiration procedures.

Preparation of myeloma cells. Myeloma cells were purified from bone marrow aspirates as described previously. Briefly, heparinized bone marrow aspirates were centrifuged at 550 g for 15 minutes on Ficoll-Hypaque gradient and the cells at the interface were collected. These cells were fractionated on Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) discontinuous gradient ranging from 20% to 90% (vol/vol) in 1.5 mL centrifuge tubes (Corning, NY). The cells banding at interfaces between 45% and 50% and between 50% and 60% were collected, and were subjected to E-rosetting. Non-rosetting cells were collected and treated with anti-Leu M1 antibody (Becton-Dickinson, Mountain View, CA) and rabbit complement (Behring, Marburg, W Germany). Purity of myeloma cells was enumerated morphologically by light microscope (Wright's stain) and immunologically by direct immunofluorescence. Thus, the purified fraction consisted of more than 90% myeloma cells and were contaminated with fewer than 1% OKT3+ T cells, 1% myeloid cells, and 1% erythroid cells. Only the samples having more than 90% enriched fraction of myeloma cells were used in this study. Cell viability was examined by trypan blue dye exclusion method and confirmed that the viability was over 90% in all cases.

In vitro proliferation activity. The myeloma cell rich fractions (4 \(\times\) 10^4 cells/200 \(\mu\)L/well) obtained as described above, were incubated in RPMI-1640 medium (Nissui, Japan) supplemented with 10% fetal calf serum (FCS, M.A. Bioproducts, Walkersville, MD) and 1 \(\times\) 10^{-3} mol/L 2-mercaptoethanol either in the absence or presence of various concentrations of human lymphoblastoid interferon (IFNa) with a specific activity of 2 \(\times\) 10^4 U/mg protein (HLBI, Sumitomo Pharmaceuticals Co, Ltd, Japan), for 48 hours in an atmosphere of 95% air and 5% CO2 at 37°C. The cells were then pulsed with 1 \(\mu\)Ci of \(^{3}H\)-thymidine (\(^{3}H\)-TdR, 5 Ci/mmol; Amersham International, England) 12 hours before harvesting; DNA synthesis was measured by \(^{3}H\)-TdR uptake.

In vitro M-protein secretion activity. After 48-hour incubation of myeloma cells as described above, culture supernatants were collected for measuring the amount of IgG or IgA by sandwich method of the enzyme linked immunosorbent assay (ELISA) as described previously. Briefly, the supernatants were diluted 1/50 to 1/200 in phosphate buffered saline (PBS), containing bovine serum albumin (100 \(\mu\)g/mL) and 0.1 mL was added to each well of microtiter plate (Falcon, Becton-Dickinson Labware, Oxnard, CA).
which were coated with goat anti-human IgG or IgA antibody (goat anti-human IgG F(ab')2, or IgA F(ab')2, TAGO, Burlingame, CA). After washing, peroxidase conjugated goat anti-human IgG or IgA (goat F(ab')2 anti-human IgG or IgA-peroxidase, TAGO) was added. 0.1% (wt/vol) 0-phenylenediamine dihydrochloride in sodium phosphate-citrate buffer containing H2O2 was added. After 20-minute incubation at 37°C, the absorbance at 405 nm was measured by a plate reader (SLT Labinstruments, Austria). The amount of IgG or IgA in the samples was determined from standard curves obtained from samples with known amount of purified human IgG or IgA (TAGO). M-protein secretion activity was measured as follows: in vitro IgG or IgA secretion rate (pg/cell/d) = concentration of IgG or IgA in the culture supernatant (ng/mL) x 10^3 / culture period (d) x myeloma cell number (cell/mL).

Ig mRNA expression. Total RNA was isolated from the cells incubated either in the absence or presence of IFNα for 24 or 12 hours by a guanidine isothiocyanate method with cesium chloride modification.13 RNA (4 µg) was subjected to electrophoresis through 1% agarose gel containing 6% formaldehyde. Gels were visualized under UV irradiation to determine the position of 28S and 18 S rRNA before transferring RNA to NYTRAN filters (Schleicher & Schunell, Inc. Keene, NH). Filters were dried and baked at 80°C for two hours in a vacuum oven, prehybridized at 42°C for 24 hours in prehybridization buffer containing 50% formamide, 5 x SSC (75 mmol/L NaCl, 75 mmol/L sodium citrate), 1 x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 10% dextran sulfate, 50 mmol/L NaPO4 buffer (pH 6.5), 1% SDS (NaDodSO4), 0.1 mg/mL denatured salmon sperm DNA, and 0.05 mg/mL tRNA, and hybridized at 42°C for 48 hours in the same buffer containing heat-denatured 7 kb fragment digested by HindIII of plasmid DNA encoding the human Ig Cy gene.14 (Japanese Cancer Research Resources Bank, Tokyo) or actin gene (Oncor, Gaithersburg, MD) radiolabeled with (α-32P)dCTP (3,000 Ci/mmol, Amersham, England) by random primer method. Filters were washed twice in 2 x SSC (300 mmol/L NaCl and 30 mmol/L sodium citrate) with 1% SDS at 25°C for 30 minutes and twice in 0.2 x SSC with 1% SDS at 60°C for 30 minutes, and were exposed at −70°C for 48 hours to Kodak XAR film.

RESULTS

The effect of IFNα on in vitro 3H-TdR uptake of myeloma cells. Myeloma rich fractions were cultured in the presence of various concentrations (0.1, 1, 10, 100 U/mL) of IFNα for 48 hours. As shown in Fig 1A, in only four cases (no. 2, 4, 6, and 11) of the 12 cases including ten cases of IgG and two cases of IgA, 3H-TdR uptakes of these myeloma cells were suppressed slightly but significantly by <100 U/mL IFNα. But in the other eight cases, 3H-uptake were not suppressed significantly. These results show that low concentrations (<100 U/mL) of IFNα have not so much inhibitory effects on myeloma cell proliferation in vitro.

The effect of IFNα on in vitro M-protein secretion of myeloma cells. The amount of secreted M-protein increased in proportion to the culture period within 72 hours in our assay system as reported previously.11 Thus, M-protein secretion was determined in culture supernatants during 48 hours.

In vitro M-protein secretions were suppressed by IFNα in all cases except one (case 1) as shown in Fig 1B. The suppression was seen even at lower concentrations (0.1 or 1 U/mL) of IFNα. The mean suppression rate was 35.2% when 10 U/mL of IFNα was added, and 41.8% when 100 U/mL of IFNα was added. The only case (case 1) that showed no suppression of M-protein secretion was the same one that showed no suppression of 3H-TdR uptake by IFNα (Fig 1A). Thus, these data show that even lower concentrations (0.1 or 1 U/mL) of IFNα have significant inhibitory effects on in vitro M-protein secretion of myeloma cells.

The effect of IFNα on the expression of immunoglobulin mRNA in myeloma cells. As described above, since the amount of M-protein was decreased in the culture supernatants of IFNα-treated myeloma cells, we next examined whether IFNα suppressed the expression of Ig mRNA in myeloma cells (IgG type). As shown in Fig 2, the expression of secretory IgG chain mRNA (1.8 kb) was suppressed by stimulation with IFNα (100 U/mL) after 12 hours (lane 3) and 24 hours (lane 4) compared with that of the medium alone (lane 2), in case 8. IFNα-induced suppression of IgG mRNA expression showed a dose-dependent manner (data not shown). This could explain the fact that, in vitro M-protein secretion of these myeloma cells was suppressed by IFNα in a dose-dependent manner as mentioned above (Fig
and one patient (case 4) was treated with IFNa alone. Bone marrow aspirations were performed before and during or after IFNa administration, and in vitro M-protein secretions of purified myeloma cells were determined by measuring the amounts of M-protein in their culture supernatants. There were significant suppressions of M-protein secretion of the myeloma cells during or after IFNa administration in vivo (Fig 3). In cases 5 and 8, serum M-protein (IgG or IgA) levels decreased in accordance with the reduction of in vitro M-protein secretion, even though in case 8, myeloma cells in

Fig 2. The effect of IFNa on the expression of Ig mRNA in myeloma cells. In one case (no. 8), purified IgG myeloma cells were cultured with IFNa (100 U/mL) for 12 hours (lane 3) and 24 hours (lane 4), or medium alone (lane 2). A human myeloid cell line (K-562) was used as control (lane 1). Four micrograms of total RNA was applied to each lane. Sizes in kilobase were estimated with reference to ribosomal RNAs. Secretory IgG mRNA (1.8 kb) was detected by using C[gamma] as a probe. The expressions of beta-actin mRNA are shown at the bottom.

In other cases (cases 6, 7), similar results were also obtained (data not shown); IFNa suppressed the expression of secretory type IgG mRNA in IgG myeloma cells. Therefore, these results show that IFNa suppresses the expression of Ig mRNA, and IFNa-induced suppression of M-protein secretion may be due to the decreased Ig mRNA expression in IFNa-treated myeloma cells.

In vitro M-protein secretion of myeloma cells during or after in vivo administration of IFNa. We examined whether IFNa suppressed M-protein secretion of myeloma cells during or after its administration in vivo. As shown in Fig 3, in the three representative cases (cases 4, 5, and 8), these patients were injected, intramuscularly, with a daily dose of 3 or 6 x 10^6 U IFNa for at least 1 month. Two of the three patients (cases 5 and 8) were treated in combination with intermittent melphalan and prednisolone (MP) therapy, and one patient (case 4) was treated with IFNa alone. Bone marrow aspirations were performed before and during or after IFNa administration, and in vitro M-protein secretions of purified myeloma cells were determined by measuring the amounts of M-protein in their culture supernatants. There were significant suppressions of M-protein secretion of the myeloma cells during or after IFNa administration in vivo (Fig 3). In cases 5 and 8, serum M-protein (IgG or IgA) levels decreased in accordance with the reduction of in vitro M-protein secretion, even though in case 8, myeloma cells in

Fig 3. In vitro M-protein secretion after treatment of myeloma patients with IFNa. Three myeloma patients (two IgG type, one IgA type) were treated with 3 x 10^6 or 6 x 10^6 U/d of IFNa. Treatment in cases no. 5 and 8 was combined with melphalan-prednisolone (MP) therapy. Case no. 4 was treated with IFNa alone. In vitro spontaneous M-protein secretions were measured as described in Materials and Methods. The results of multiple frozen aliquots of several myeloma cell preparations varied by <10% from the mean. In the figures, columns represent the relative nuclear cell counts in the bone marrow, and shaded areas show the percentage of myeloma cells in the bone marrow.
the bone marrow increased after IFN administration. In case 4, after IFNα therapy, in vitro M-protein secretion decreased, but serum M-protein (IgG) level remained unchanged. As reported previously, serum M-protein level could be regulated by many factors: myeloma cell number, M-protein synthesis rate, M-protein metabolism rate, and so on. These data suggest that IFNα suppressed M-protein secretion of myeloma cells in vivo as well as in vitro.

With regard to the case (case 1) that showed no suppression of ³H-TdR uptake and no suppression of in vitro M-protein secretion, the profile of the case was a 68-year-old woman, IgGA type, and clinical stage II A. This case showed no clinical response to IFNα, i.e., no reduction of the serum IgG had been observed 6 months after 1 month administration of IFNα (3 x 10⁶, daily).

**DISCUSSION**

Our data presented in this report demonstrate that IFNα suppressed M-protein secretion of myeloma cells, in vivo as well as in vitro. The inhibitory effect of IFNα on M-protein secretion was more pronounced than on in vitro proliferation of myeloma cells, i.e., at as low as 0.1 U/mL concentration, IFNα suppressed significantly in vitro M-protein secretion of myeloma cells, while the in vitro proliferation was not so suppressed. Furthermore, the data demonstrated that in vitro M-protein secretion of myeloma cells in myeloma patients were actually suppressed during or after IFNα administration in vivo. Brenner et al reported that IFNα or recombinant IFNα inhibited proliferation and concomitantly decreased the rate of M-protein secretion in cultures of the myeloma cell line that was sensitive to IFNα, but not in those of IFN-resistant cell line. In addition, IFNα at the concentration of 100 U/mL was shown to decrease M-protein production without exerting more than minimal cytotoxicity on the IFN-sensitive cell line. It was also reported previously that when IFNα was administered intramuscularly at 3 to 6 x 10⁶ U daily, serum concentration of IFNα was not more than 100 U/mL. Therefore, it is likely that M-protein secretion of myeloma cells could be suppressed significantly, in vivo, even when myeloma cell proliferation could not be so suppressed, by IFNα administration in a daily dose of 3 to 6 x 10⁶ U, and thus, in some cases, serum M-protein levels might be decreased without the reduction of the myeloma cell mass. In clinical aspects, this is an important point to be considered when evaluating clinical response to IFNα in myeloma patients, where the response to the treatment is evaluated mainly by the reduction of serum M-protein or urinary excretion of M-protein.

On the other hand, the precise mechanism of the suppressive effects by IFNα is still unclear. There are reports demonstrating that treatment of lymphoblasts Daudi cells with IFNα caused a significant decrease in the level of c-myc mRNA, and its suppressive effect was selective in c-myc mRNA expression and IFNα treatment did not appear to affect the c-myc transcription rate, but rather reduced the half-life of its mRNA. Thus, the mechanism of antiproliferative effect by IFNα in myeloma cells remains to be clarified. As for the suppressive effect of IFNα on protein synthesis, Goldring et al reported that IFNα suppressed collagen synthesis and procollagen mRNA levels.

In this report, we have demonstrated that IFNα suppressed M-protein (IgG or IgA) secretion of myeloma cells, and furthermore, IFNα selectively suppressed the expression of secretory Ig mRNA. But it is still unclear whether IFNα reduces either the Ig transcription rate, or the stability of Ig mRNA.

The regulatory mechanism of proliferation and M-protein secretion in myeloma cells is an important issue to be investigated. As reported previously, myeloma cells constitutively produce IL-6 and express IL-6 receptors, and myeloma cells respond to IL-6 to proliferate. There is a possibility that IFNα modulates IL-6-induced proliferation mechanism; IFNα may reduce IL-6 production or suppress IL-6 receptor expression. These studies are now in progress. In our study, this is also the case in that IFNα selectively suppresses the expression of Ig mRNA. However, it remains to be clarified how IFNα-induced signals reduce the expression of Ig mRNA.

In this report, we conclude that IFNα has more profound inhibitory effects on M-protein secretion, of human myeloma cells, than on myeloma cell proliferation. Further studies on the suppressive mechanism of IFNα will provide us with more important information for the accurate assessment of clinical response to IFNα, as well as institution of an appropriate treatment schedule using IFNα in myeloma patients.

**ACKNOWLEDGMENT**

We thank Dr. D.M. Matsuwa for critical review of this manuscript, and H. Sumida, Y. Ohto, and K. Yamamoto for their excellent secretarial assistance.

**REFERENCES**

5. Quesada JR, Hawkins M, Hornig S, Alexanian R, Borden E, Merigan T, Adams F, Guttermann JU: Collaborative phase I-II study of recombinant DNA-produced leukocyte interferon (clone A) in...
Sensitive inhibitory effect of interferon-alpha on M-protein secretion of human myeloma cells

H Tanaka, O Tanabe, K Iwato, H Asaoku, H Ishikawa, M Nobuyoshi, M Kawano and A Kuramoto