Effect of Glucocorticoids on the Biologic Activities of Myeloma Cells: Inhibition of Interleukin-1β Osteoclast Activating Factor-Induced Bone Resorption

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

Regulatory effects of glucocorticoids (dexamethasone) on myeloma cells as well as bone resorption in multiple myeloma were investigated. Glucocorticoids significantly inhibited proliferation of myeloma cells, and decreased the messenger RNA (mRNA) expressions of interleukin-6 (IL-6) and secretory type immunoglobulin G (IgG). The inhibitory effects of glucocorticoids on myeloma cell proliferation could be due to the decreased expression of IL-6 mRNA, decreased IL-6 production, and thus suppression of autocrine growth by IL-6, which is an autocrine growth factor for myeloma cells as reported previously (Nature 332:83, 1988). Glucocorticoids also inhibited M-protein secretion by decreasing the levels of secretory type Ig mRNA. On the other hand, because IL-1β rather than lymphotxin is considered to be a major osteoclast activating factor (OAF) produced by myeloma cells, and glucocorticoids decreased the expression of IL-1β mRNA and markedly suppressed the bone resorbing activity induced by IL-1β OAF in 45Ca-release bone resorption assay, it is suggestive that glucocorticoids could inhibit bone resorption induced by IL-1β OAF in multiple myeloma. Therefore, from these data it is concluded that glucocorticoids could be more effective chemotherapeutic agents in multiple myeloma than we expected, especially with regards to the inhibitory effects on proliferation and M-protein secretion from myeloma cells, as well as bone resorption by myeloma cells.

MATERIALS AND METHODS

Reagents and cDNA. Dexamethasone was purchased from Sigma (St. Louis, MO). Recombinant human IL-1β and human IL-12 cDNA were kindly provided by Dr Y. Hirai (Otsuka Pharmaceutical Co, Ltd, Tokushima, Japan). Human IL-6 cDNA was kindly provided by Drs T. Hirano and T. Kishimoto (Osaka University, Osaka, Japan). Human immunoglobulin G (IgG) cDNA was a gift from Japanese Cancer Research Resources Bank (Tokyo, Japan). Calcitonin (Ecatonin) and synthetic human parathyroid hormone (PTH)-(1-34) (6,000 U/mg) were provided by Toyo Jozo Co (Tokyo, Japan).

MATERIALS AND METHODS

MULTIPLE MYELOMA (MM) is a hematologic disorder characterized by clonal proliferation of malignant plasma cells that secrete monoclonal immunoglobulins (M-protein), and also has a prominent feature of bone lytic lesions.1 Recently, a regulatory mechanism of myeloma cell proliferation has been reported indicating interleukin-6 (IL-6) to be an autocrine growth factor for myeloma cells; i.e., myeloma cells constitutively produce IL-6 and express IL-6 receptor,2 and that IL-1 accelerates autocrine growth of myeloma cells through IL-6.3 On the other hand, an osteolytic mechanism in myelomas has further been shown to be involved through an osteoclast activating factor (OAF) produced by the myeloma cells, although the OAF(s) produced by these myeloma cells is still controversial. Garrett et al4 reported that lymphotxin was OAF from myeloma cells, but Kawano et al5 found that IL-1 activities were detectable in the culture supernatants of freshly isolated myeloma cells, and that IL-1β rather than lymphotxin exhibited the major bone resorbing activity in MMs.

In this report, to re-evaluate the effectiveness of glucocorticoids that have been used as chemotherapeutic agents in MM,6,7 we investigated the effects of glucocorticoids on the proliferation of myeloma cells, expression of secretory type Ig, mRNA, and bone resorption induced by IL-1β OAF. Our results showed that glucocorticoids not only have anti-proliferative effects on myeloma cells, partly through the suppression of IL-6 production, but also have anti-osteolytic effects evidenced by inhibiting IL-1β production and simultaneously blocking osteoclast-activation by the secreted IL-1β and its subsequent effect on bone resorption. Thus, we propose that glucocorticoids could be more effective chemotherapeutic agents in multiple myeloma than we expected.

In vitro proliferation activity of myeloma cells. Myeloma cell-rich fractions, obtained as described above, were cultured at 4 x 10⁶ cells/mL in 0.2 mL of RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; M.A. Bioproducts, Walkersville, MD) and 1 x 10⁻³ mol/L 2-mercaptoethanol (2-ME) in the presence of 10⁻⁷, 10⁻⁸, or 10⁻⁹ mol/L dexamethasone for 48 hours in a humidified 5% CO₂ atmosphere at 37°C. Cells were pulsed with 1 μCi of ³H-thymidine (³H-Tdr, 5 Ci/mmol, Amersham, Buckinghamshire, UK) 12 hours before harvesting, and DNA synthesis was measured by ³H-Tdr uptake. IgG, IL-6, and IL-1β mRNA expression. Total RNA was

From the Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Hiroshima; and the Division of Internal Medicine, Kyoto City Hospital, Kyoto, Japan.

Submitted May 16, 1989; accepted September 21, 1989.

Supported in part by grants from the Ministry of Education Science and Culture, and the Naito Foundation.

Address reprint requests to Michio Kawano, MD, Myeloma Study Group, Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Kasumi 1-2-3, 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.

© 1990 by The American Society of Hematology.
isolated from myeloma cells after 24-hour culture in the presence of 10^{-4} or 10^{-5} mol/L dexamethasone by a guanidine isothiocyanate method with cesium chloride modification.\(^1\) RNA (10 \(\mu\)g) was subjected to electrophoresis through 1% agarose gel containing 6% formaldehyde. After electrophoresis, RNA was transferred to NYTRAN filter (Schleicher & Schuell, Keene, NH). Filters were dried; baked at 80°C for 2 hours in vacuo; prehybridized at 42°C for 24 hours in a prehybridization buffer containing 50% formamide, 5X SSC, 1X Denhardt’s solution, 10% dextran sulfate, 50 mmol/L sodium phosphate buffer (pH 6.5), 1% SDS, 0.1 mg/mL denatured salmon sperm DNA, and 0.05 mg/mL tRNA; and then hybridized at 42°C for 48 hours in the same buffer containing a heat-denatured plasmid DNA encoding human IgG, IL-6, and IL-1β radiolabeled with \([^{3}P]dTTP (3,000 \text{ Ci/mmol, Amersham})\) by a random primer method. Filters were washed twice in 2X SSC at 1% SDS at 25°C for 30 minutes and twice in 0.2X SSC and 1% SDS at 60°C for 30 minutes, and were exposed at -70°C to Kodak XAR film (Eastman Kodak, Rochester, NY).

**Bone resorption assay.** Bone resorbing activity was measured by a method described previously.\(^4\) In brief, 2-day-old mice (C3H-ATD) were injected with \(1 \mu\)Ci of \(^{45}CaCl_2\) (Amersham). Two days later, the parietal bones were dissected and each bone was incubated in Eagle Minimal Essential Medium (Nissui, Tokyo, Japan) supplemented with 5% horse serum (GIBCO, Grand Island, NY). Twenty-four hours later, the medium was changed to a fresh one with or without the test substance. Culture was continued for the following 48 hours, and the bone pieces and the culture supernatants were removed. The radioactivities in the bone pieces and culture supernatants were measured in a \(\beta\)-scintillation counter (Tri-Carb 460 C, Packard, IL). Bone resorbing activity was expressed as the percentage of \(^{45}Ca\) released or as the ratio of the \(^{45}Ca\) released from the treated bones to the amount released from corresponding control bones. At least six bone pieces were used for each concentration of the tested substance. Statistical significance was evaluated by the Student’s \(t\) test.

**RESULTS**

**Effect of dexamethasone on in vitro proliferation of myeloma cells.** Myeloma-rich fractions were cultured in the presence of various concentrations of dexamethasone (10^{-7}, 10^{-6}, or 10^{-5} mol/L) for 48 hours. As shown in Fig 1, \(^{3}H\)-TdR uptakes of these myeloma cells were suppressed significantly in a dose-dependent manner. A similar phenomenon was found when myeloma cells were cultured for 24 of 72 hours (data not shown). It should be noted that the inhibitory effects of dexamethasone on myeloma cell proliferation depended on myeloma cells isolated from various cases of myelomas. Dexamethasone also suppressed IL-6-induced proliferation of myeloma cells in a dose-dependent manner. On the other hand, exogenous excessive amounts of IL-6 overcame the anti-proliferative effects of dexamethasone (data not shown). Calcitonin had no inhibitory effects on the proliferation of myeloma cells that we tested (data not shown). These results show that dexamethasone or glucocorticoids have an inhibitory effect on proliferation of myeloma cells.

**Effect of dexamethasone on the expression of IL-6, IgG, and IL-1β mRNA in myeloma cells.** Dexamethasone inhibited an in vitro proliferation of myeloma cells as mentioned above, whereas IL-6 is an autocrine growth factor for myeloma cells. Thus, this situation is suggestive of a possibility that dexamethasone inhibited production of IL-6 in these myeloma cells. To confirm this, we investigated whether dexamethasone decreased the expression of IL-6 mRNA. As shown in Fig 2A, dexamethasone markedly decreased the expression of IL-6 mRNA when the myeloma cells were cultured for 24 hours with 1 \(\times\) 10^{-4} mol/L dexamethasone. This was also the case when myeloma cells were cultured for 48 hours or with 1 \(\times\) 10^{-5} mol/L dexamethasone (data not shown).

Dexamethasone also suppressed the expression of secretory type IgG mRNA in myeloma cells (Fig 2B). This could also be explained in this way: dexamethasone inhibited M-protein secretion of myeloma cell.

Furthermore, IL-1β rather than lymphotoxin is a major OAF in myelomas, as reported previously,\(^2\) and IL-1β as well as IL-1α also accelerate autocrine growth of myeloma cells through IL-6.\(^3\) Thus, we investigated the effect of dexamethasone on the expression of IL-1β mRNA in myeloma cells. The expression of IL-1β mRNA in myeloma cells was markedly suppressed when myeloma cells were cultured for 24 hours with 1 \(\times\) 10^{-6} mol/L dexamethasone as shown in Fig 2C. Similar results were also obtained when myeloma cells were cultured for 48 hours or with 1 \(\times\) 10^{-7} mol/L dexamethasone (data not shown). It is not likely that the suppressive effect of dexamethasone could have been derived from the cytotoxic effect on myeloma cells because viability of these myeloma cells, determined by trypan blue dye exclusion after 24- or 48-hour culture with dexamethasone (1 \(\times\) 10^{-6} or 1 \(\times\) 10^{-7} mol/L), was not significantly altered. In addition, IFNα suppressed the expression of IL-6 and IgG, but not IL-1β mRNA, as described previously.\(^15\)

On the other hand, calcitonin had no significant effects on the expression of the mRNAs in these myeloma cells. These
Fig 2. Effect of dexamethasone on the expression of IL-6, IgG, and IL-1β mRNA in myeloma cells. Ten micrograms of total RNA was applied to each lane. Sizes in kilobases (kb) were estimated with reference to ribosomal RNAs. (A) Effect of dexamethasone on the expression of IL-6 mRNA in myeloma cells. Normal monocyte stimulated by lipopolysaccharide (LPS) was used as positive control (lane 1). Freshly isolated myeloma cells are seen in lane 2. Purified myeloma cells were cultured for 24 hours with medium alone (lane 3); interferon (IFN) (100 U/mL) (lane 4); IL-1α (10^3 U/mL) (lane 5); IL-6 (20 U/mL) (lane 6); or dexamethasone (10^{-6} mol/L) (lane 7). IL-6 mRNA (1.0 kb) was detected by using IL-6 cDNA (Taq I/Ban II fragment of pBSF 2.381) as a probe. (B) Effect of dexamethasone on the expression of IgG mRNA in myeloma cells. A human myeloid cell line (K-562) was used as negative control (lane 1). Purified myeloma cells were cultured for 48 hours with medium alone (lane 2). Purified myeloma cells were cultured for 12 hours (lane 3) or 24 hours (lane 4) with IFNα (100 U/mL), or cultured for 48 hours with IL-1α (10^3 U/mL) (lane 5); IL-6 (100 U/mL) (lane 6); or dexamethasone (10^{-6} mol/L) (lane 7). IgG mRNA (1.8 kb) was detected by using Cγ as a probe. (C) Effect of dexamethasone on the expression of IL-1β mRNA in myeloma cells. Normal monocyte stimulated by LPS was used as a positive control (lane 1). Purified myeloma cells were cultured for 24 hours with medium alone (lane 2); IFNα (100 U/mL) (lane 3); IL-1α (10^3 U/mL) (lane 4); IL-6 (100 U/mL) (lane 5); dexamethasone (10^{-6} mol/L) (lane 6); or calcitonin (1 mU/mL) (lane 7). IL-1β mRNA (1.6 kb) was detected by using IL-1β cDNA as a probe.
data showed that dexamethasone significantly suppressed the expression of IL-6, IgG, and IL-1β mRNA in myeloma cells.

**Effect of dexamethasone on IL-1β (OAF)-induced bone resorption.** Dexamethasone suppressed the expression of IL-1β mRNA in myeloma cells as shown above. Thus, we investigated the effect of dexamethasone on bone resorption induced by IL-1β (OAF). Dexamethasone inhibited bone resorption induced by culture supernatants of myeloma cells (case 1, IgG-, stage IIIA, multiple bone lytic lesions; case 2, BJP-, stage IIIB, multiple bone lytic lesions) as shown in Fig 3. Dexamethasone itself had no effect on spontaneous bone resorption. Bone resorbing activity in the culture supernatants has been shown to be derived mainly from IL-1β; i.e., the bone resorbing activity was completely blocked by anti–IL-1β antibody. This is a confirmation of the finding that dexamethasone inhibited bone resorption induced by the recombinant IL-1β.

On the other hand, parathyroid hormone (PTH) had a strong bone resorbing activity, and dexamethasone significantly augmented the effect of PTH on bone resorption. On the contrary, calcitonin markedly suppressed bone resorption induced by culture supernatants from myeloma cells.

Therefore, these results show that dexamethasone inhibits bone resorption induced by IL-1β (OAF) as well as IL-1β production in the myeloma cells.

**DISCUSSION**

The main finding reported here is that glucocorticoids (dexamethasone) inhibited proliferation and M-protein secretion of myeloma cells by suppressing IL-6 mRNA expression and decreasing the levels of secretory type Ig mRNA, respectively. It also inhibited IL-1β production in myeloma cells as well as bone resorption induced by IL-1β OAF. These were also confirmed that IL-1 activity (mouse thymocyte comitogenic assay), IL-6 activity (IL-6–dependent mouse hybridoma MH60, BSF2 proliferation assay), and IgG level (enzyme-linked immunosorbent assay) were decreased in the culture supernatants of dexamethasone-treated myeloma cells (data not shown). In these experiments, the suppressive dose (10⁻⁵ to 10⁻⁷ mol/L) of dexamethasone was in the pharmacologic range.

Glucocorticoids have been reported to modulate mRNA expression of eukaryotic genes; glucocorticoids increased the mRNA expression of mammary tumor virus, growth hormone in rat pituitary cells, metallothioneins in mouse liver, tyrosine aminotransferase in rat liver, α2u globulin in transfected mouse cells, and ovalbumin in chick oviduct; whereas they decreased the mRNA expression of c-myc, c-myb, and c-Ki-ras in mouse lymphoid cells, adrenocorticotropic in rat pituitary glands, α-fetoprotein in developing placenta, IFN-γ, procollagen type I in fibroblast cell lines, and glucocorticoid receptors. Recent works show that glucocorticoid receptors have DNA binding domains and genes to be regulated by glucocorticoids have glucocorticoid responsive elements (GRE) in their 5' upstream or introns; thus, when glucocorticoid receptors bind the GRE, gene expressions are modulated. The genes of IL-1α and IL-1β have parts of consensus GRE sequences at intron 6 and intron 5, respectively. The IL-6 gene has GRE sequences in the 5' flanking region. Therefore, it is relevant to expect that glucocorticoids inhibit the gene expression of IL-1 and IL-6 at the transcriptional level. Recent reports show that glucocorticoids selectively inhibit IL-1 gene expression at the transcriptional level. The precise mechanism of IL-6 gene regulation by glucocorticoids remains to be clarified.

Glucocorticoids inhibited bone resorption induced by IL-1β (OAF). Although it is still unclear whether IL-1 can act on osteoclast or not, osteoblasts but not osteoclasts have glucocorticoid receptors. Thus it is likely that glucocorticoids have some effects on osteoblasts and indirectly inhibit functions of osteoclast. On the other hand, glucocorticoids upregulated the effect of PTH: glucocorticoids were reported to augment the expression of PTH receptors on osteoblasts. Bone resorption induced by PTH and accelerated in the presence of glucocorticoids could be completely inhibited by calcitonin. Calcitonin has direct effect on osteoclasts and inhibits bone resorption, but has no effect on myeloma cells: proliferation and M-protein secretion of myeloma cells were not affected by calcitonin, and calcitonin could not modulate the expression of IL-6 and IL-1β mRNA in myeloma cells.
In this context, glucocorticoids are useful chemotherapeutic agents that inhibit bone resorption in myelomas, particularly when calcitonin is used in combination with glucocorticoids; glucocorticoids suppressed not only the expression of IL-1β (OAF) mRNA in myeloma cells, but also bone resorption induced by IL-1β. Furthermore, calcitonin completely inhibited PTH-induced bone resorption that was augmented by glucocorticoids.

Therefore we conclude that glucocorticoids are more effective chemotherapeutic agents in multiple myeloma than what we have always expected, and the regulatory mechanism of glucocorticoids on IL-6 gene expression could contribute to the understanding of transcriptional regulation of IL-6 gene activation.

ACKNOWLEDGMENT

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

REFERENCES
hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. Proc Natl Acad Sci USA 83:5899, 1986


37. Lew W, Oppenheim JJ, Matsushima K: Analysis of the suppression of IL-1α and IL-1β production in human peripheral blood mononuclear adherent cells by a glucocorticoid hormone. J Immunol 140:1895, 1988


40. Yamamoto I, Potts JT, Segre GV: Glucocorticoids increase parathyroid hormone receptors in rat osteoblastic osteosarcoma cells (ROS 17/2). J Bone Min Res 3:707, 1988