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Gamma interferon down-regulates glucocorticoid receptor expression and attenuates hormone action in a human osteosarcoma cell line

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The effect of gamma interferon (IFN) on glucocorticoid receptor (GR) expression was studied in HOS-8603 cells, a human osteogenic sarcoma cell line. Treatment of HOS-8603 cells with IFN resulted in down-regulation of GR number, with no change in the binding affinity for glucocorticoids. The maximum decrease in receptor binding was evident at 10 IU/ml IFN concentration. Time-course studies revealed that the effect reached a maximum at 36 h treatments. To clarify the molecular basis for the down-regulation of GR by IFN, change in GR mRNA levels was further investigated by RNA blot hybridization analysis. It was found that there also existed a time-dependent decrease in GR mRNA levels in HOS-8603 cells after treatment with IFN. In the presence of IFN, the inhibitory effect of glucorticoids on HOS-8603 cell proliferation was blunted. Moreover, the induction of alkaline phosphatase (AKP) activity by glucocorticoids was attenuated in response to IFN treatment. These data suggest that IFN may influence GR activity which at least partially occurs at mRNA levels, and that the decrease in receptor activity in HOS-8603 cells parallels with the decrease in glucocorticoid-mediated functional responses.

Keywords: glucocorticoid; glucocorticoid receptor; gamma interferon; down-regulation; alkaline phosphatase; osteosarcoma cell line (human)

Introduction

Glucocorticoid hormones affect the differentiation, development, and growth of a wide range of eukaryotic cells and tissues, eliciting a variety of catabolic and anabolic responses (Yamamoto, 1985; Cook *et al.*, 1988; Evans, 1988). The effects of glucocorticoids are manifested at the molecular level by stimulation and/or repression of target genes in specific tissues. The cascade of events leading to the induction of gene expression by glucocorticoids begins with the lipophilic hormone crossing the plasma membrane of a target cell and binding with high affinity to the glucocorticoid receptor (GR). When the hormone binds to the receptor, the hormone-receptor complex interacts with specific cis-acting regulatory DNA sequences known as glucocorticoid response elements (GREs) located in the vicinity of the regulated gene (Beato, 1989; Fuller, 1991). Once bound to the GREs, the GR is thought to interact with other components of the transcription apparatus to either enhance or repress the expression of the linked gene. Several lines of evidence indicate that intracellular concentration of GR is an important factor in determining the extent of the biological response of target cells to glucocorticoids. It has been reported that there is a strong correlation between the intracellular concentration of GR and the cytolytic response of murine lymphoma cells to glucocorticoids (Bourgeois & Newby, 1979; Danielson & Stallcup, 1984; Gehring *et al.*, 1984). It has been shown more directly that, after transfection of the GR gene into cells lacking functional GR, the magnitude of the transcriptional response from either a cotransfected reporter gene (Giguere *et al.*, 1986) or the endogenous tyrosine aminotransferase gene (Vanderbilt *et al.*, 1987) is proportional to the level of GR expression achieved. It is therefore, possible that regulation of GR levels may be a physiological means of modulating cellular responses to glucocorticoids.

There is considerable evidence that GR levels in cells are regulated by glucocorticoid hormones (Okaley & Cidlowski, 1993). Glucocorticoids have been found to down-regulate the levels of their own receptor in a number of different cell lines (Cidlowski & Cidlowski, 1981; Svec & Rudis, 1981; Lacroix *et al.*, 1984; McIntyre & Sammuels, 1985), in target issues of intact rats (Tornello *et al.*, 1982; Sapolsky *et al.*, 1984; Yang *et al.*, 1989) and in lymphocytes of steroid-treated human volunteers (Schlecte *et al.*, 1982). More recently, GR levels have been shown to be up-regulated by glucocorticoids in a human leukemia cell line, suggesting that the pattern of homologous GR regulation may be tissue specific (Eisen *et al.*, 1988). However, little is known about the heterologous regulation of GR. The best described model is probably the up-regulation of GR by cAMP. Based on ligand-binding assay, Oikarinen *et al.* (1984) showed that cAMP stimulated while cGMP inhibited glucocorticoid binding in cultured human skin fibroblast cells. Gruol *et al.*, (1986) also found that cAMP increased the level of glucocorticoid binding in murine lymphoma cells. This increase in glucocorticoid binding required functional cAMP-dependent protein kinase activity, since cells containing reduced kinase activity showed impaired response to cAMP. More recently, Dong *et al.* (1989) further showed that the up-regulation of GR by cAMP correlated well with the increase in inducibility of two glucocorticoid regulated genes, and that the increased GR was at least in part caused by GR mRNA stability.

IFN, as well as having antiviral activity, also exhibits various biological activities, such as antitumor activity (Czarnieckl *et al.*, 1984), modulation of cell growth and differentiation (Rossi, 1985). It has been reported that IFN inhibited, did not affect, or enhanced the growth of different osteosarcoma cell line (Harju *et al.*, 1990; Tong *et al.*, 1992). Furthermore, IFN has also been reported to suppress osteosarcoma growth in mice (Satomi, 1983) and to suppress metastasized osteosarcoma in the lung (Ito *et al.*, 1980). In the present study, the effect of IFN on the proliferation of HOS-8603, a newly established human esteogenic cell line was initially studied. It was found that IFN alone did not affect HOS-8603 cell growth in a wide range of concentrations either in monolayer culture or in methylcellulose culture systems. However, IFN could significantly result in an attenuation of the amplitude of the glucocorticoid-induced suppression of HOS-8603 cell clonal proliferation. Further studies demonstrated that the treatment of HOS-8603 cells with IFN induced a decrease in GR binding in a dose- and time-dependent manner and that the change in GR binding was explained at least in part by a decreased GR mRNA level. Finally, the significance of IFN-induced down-regulation of GR in HOS-8603 cells was also evaluated.

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Results

Effects of IFN and Dex on HOS-8603 cell proliferation

When HOS-8603 cells in exponential growth phase were cultured in medium supplemented with 5% DCC-FCS, treatment with Dex resulted in a marked concentration-dependent decrease in cell number. Maximal growth inhibition was accompanied by a reduction in cell number to about 45% of controls (Figure 1). In contast, when HOS-8603 cells were treated with 0 to 1000 IU/ml of IFN, the proliferation of these cells were not affected at any doses (data not shown). However, when HOS-8603 cells were treated with various concentrations of Dex in combination with 10 IU/ml IFN, the inhibitory effect of Dex was significantly attenuated (Figure 1). To clarify whether the endogenous glucocorticoids from serum could interfere with the results, experiments were also carried out with serum untreated with DCC, and similar results were obtained (data not shown). Therefore, the following studies were carried out with either FCS or DCC-FCS in the culture medium as indicated.

Anchorage-independent growth of HOS-8603 cells in methylcellulose culture system was also assessed. In these culture systems, about 24% of originally seeded cells formed large colonies in untreated controls while only about 6% of originally seeded cells could form large colonies in the presence of 10 nM Dex. Even though 10 IU/ml IFN alone did not affect the colony forming ability of HOS-8603 cells, it could significantly attenuate the effects of Dex on clonal proliferation of HOS-8603 cells. These results were depicted in Figure 2.

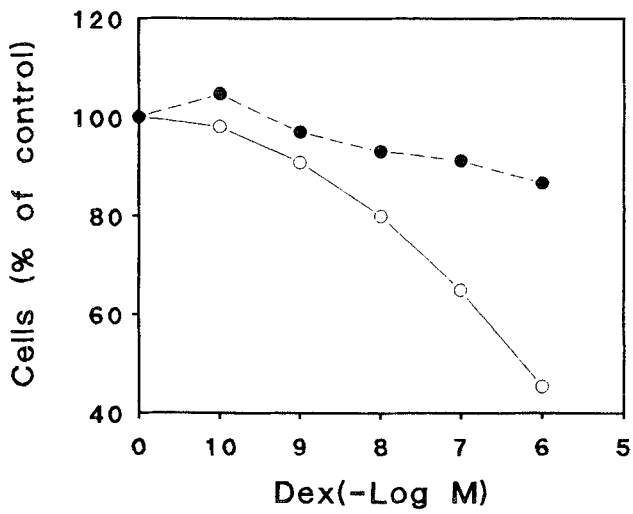

Figure 1 Effects of Dex and IFN on HOS-8603 cell proliferation. HOS-8603 cells (2×10^4 well) were plated onto 24-well plates in culture medium supplemented with 5% DCC-FCS. Twenty-four hours later, the culture medium was replaced by fresh medium containing increasing concentrations of Dex (○) or Dex + 10 IU/ml IFN (●). On day four, the number of cells per well was counted. Data are means of triplicate assays and expressed as a percentage of control cultures

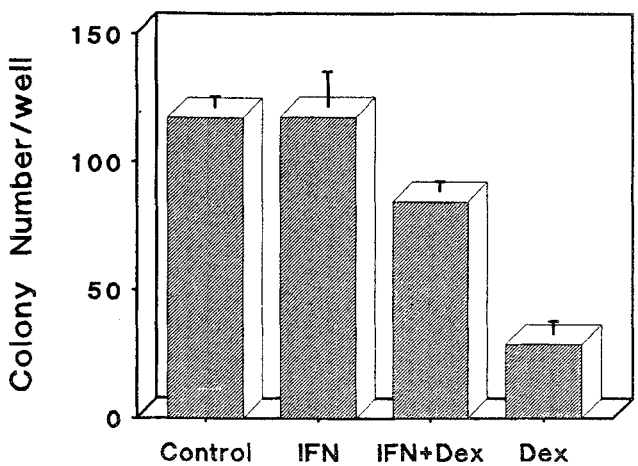

Figure 2 Effects of IFN and Dex on clonal proliferation of HOS-8603 cells. Five hundred cells were cultured with 10 nM Dex, 10 IU/ml IFN or both in RPMI 1640 containing 0.8% methylcellulose and 15% FCS. After 12 days of culture, colonies were scored under an inverted microscope. Values represent mean ± SD of six determinations. $P<0.001$ for Dex and IFN + Dex *vs* control; $P<0.01$ for Dex *vs* IFN + Dex

Effects of IFN on GR binding in HOS-8603 cells

HOS-8603 cells were treated with 10 IU/ml IFN for 48 h, following which GR binding was measured by saturation analysis and corresponding Scatchard plot. As shown in Figure 3, the binding capacity of GR in IFN-treated cells was significantly decreased relative to controls. In contrast, the K_d value for binding in IFN treated cells was not different from that in untreated controls.

Time-course of the effect of IFN on GR binding

The results of the time of incubation of HOS-8603 cells with 10 IU/ml IFN are illustrated in Figure 4. In these experiments, HOS-8603 cells were exposed to 10 IU/ml IFN for the indicated times, and the GR was assessed using a whole cell, one point binding assay. GR binding decreased to about 60% of control 24 h after IFN treatment and maximal reduction of about 50% was achieved at 36 h.

Dose effect of IFN on GR binding

To assess the sensitivity of HOS-8603 cells to IFN reduction of GR, a dose-response analysis was performed. In these experiments, HOS-8603 cells were treated with medium or increasing concentrations of IFN for 48 h. As depicted in Figure 5, binding of [^{3}H] Dex to GR decreased slightly with 1 IU/ml IFN, reaching a maximum at 10 IU/ml.

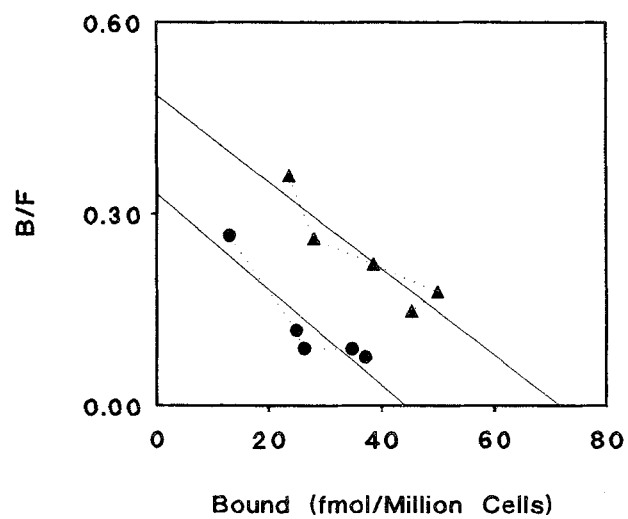

Figure 3 Scatchard analysis of [^{3}H] Dex binding in HOS-8603 cells untreated (triangle) or treated with 10 IU/ml IFN (circle) for 48 h. For Scatchard analysis, cells were incubated with varying concentrations of [^{3}H] Dex as described in the Materials and methods. Specific binding derived by subtracting nonspecific from total binding was used to generate the Scatchard plots. Each point represents the average of duplicates. Similar results were obtained in three experiments

Effects of IFN or GR mRNA

To determine whether the changes in GR binding were due to changes in steady state levels of GR mRNA, a dot blot analysis was carried out. As shown in Figure 6, GR mRNA was decreased significantly 24 h after treatment of HOS-8603 cells with 10 IU/ml IFN and the lowered level of GR mRNA lasted for up to 48 h, suggesting that IFN down-regulates GR at least partially by decreasing steady state GR mRNA levels.

Effects of IFN on Dex-induced AKP activity

Our recent findings showed that glucocorticoids could significantly induce the AKP, an enzyme marker of osteoblastic phenotype, in HOS-8603 cells in addition to inhibiting cell growth (Song, 1994). To elucidate the relationship between changes in cellular GR level induced by IFN and biological response to glucocorticoid administration, the AKP activity in HOS-8603 cells in response to Dex, INF, or both was determined. In these experiments, HOS-8603 cells were incubated with Dex, in the presence or absence of 10 IU/ml IFN for up to 72 h. Cellular AKP activity was determined as described in Materials and methods. Figure 7 showed that AKP activity in HOS-8603 cells was increased in a time-dependent manner, reaching a maximal induction at 72 h, while IFN alone did not have any effect on AKP activity. However, the combination of IFN and Dex significantly attenuated the glucocorticoid-induced increase in AKP activity.

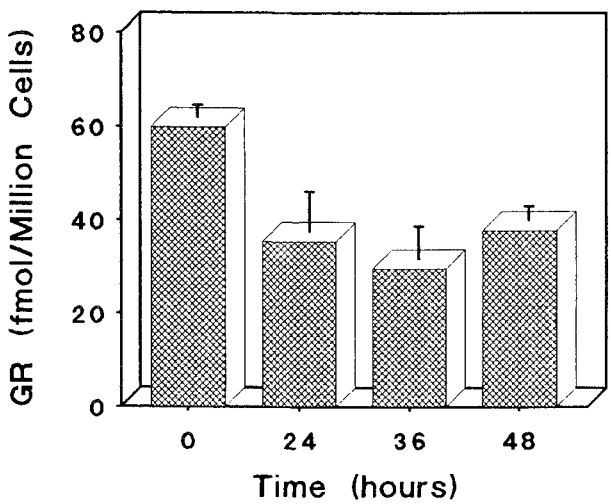

Figure 4 Time course of IFN down-regulation of GR in HOS-8603 cells. Cells were treated with 10 IU/ml IFN for the times indicated. Cells were washed with phosphate-buffered saline and suspended in serum-free medium. Cell suspensions were incubated with [^{3}H] Dex as described in the text. Data are expressed as mean ± SD ($n = 3$). Statistical significance was evaluated by Students t-test. $P < 0.01$ for 24, 36 and 48 h *vs* control

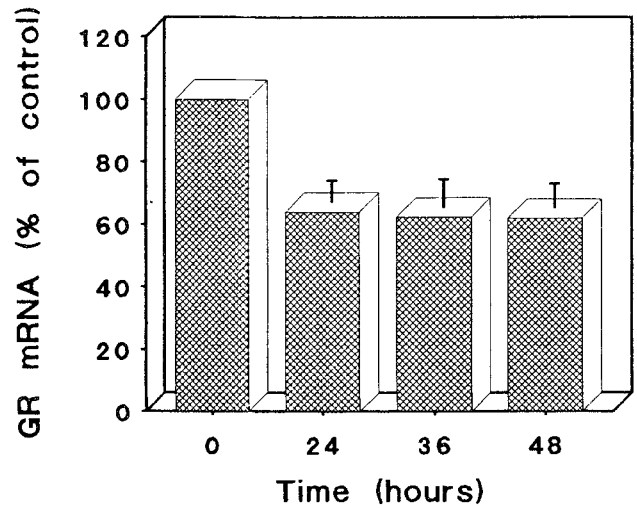

Figure 6 Effects of IFN on GR mRNA levels in HOS-8603 cells. HOS-8603 cells were treated with 10 IU/ml IFN for 0–48 h. Total RNA was isolated and used in dot blot assays. The expression level was measured by densitometric scanning, and was expressed relative to β-actin expression levels. The level of GR mRNA expression in untreated controls was arbitrary set at 100% and data represent mean ± SD of three separate experiments. $P < 0.05$ for 24 h, 36 h and 48 h *vs* 0 h, respectively

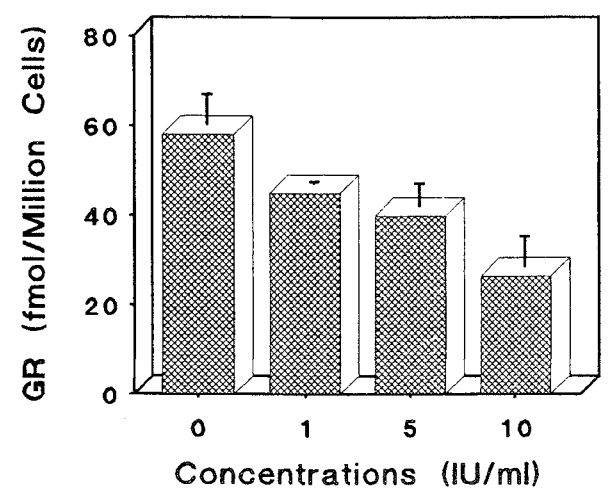

Figure 5 Dose-response of IFN down-regulation of GR in HOS-8603 cells. HOS-8603 cells were treated with or without the indicated doses of IFN for 48 h. GR levels were measured as described in the text. Data are expressed as mean ± SD ($n = 3$). Statistical significance was evaluated by Student's t-test. $P < 0.05$ for 1 and 5 IU/ml *vs* control; $P < 0.01$ for 10 IU/ml *vs* control

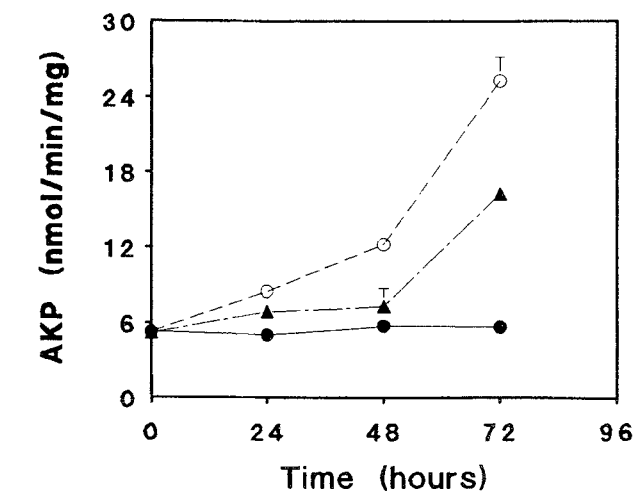

Figure 7 Effects of IFN on Dex-inducible AKP activity in HOS-8603 cells. Cells were cultured in medium containing 5% DCC-FCS in the presence of 100 nM Dex (○), 10 IU/ml IFN (●), or Dex + IFN (▲) for the time indicated. AKP activity was assayed as described in Materials and methods. Values are expressed as the mean ± SD of three independent determinations. $P < 0.05$ for Dex *vs* Dex + IFN at 24 h; $P < 0.01$ for Dex *vs* Dex + IFN at 48 and 72 h, respectively

Discussion

In this study we showed that treatment of HOS-8603 cells with IFN at very low concentrations resulted in a signficant decrease in GR binding without altering the binding affinity and that the observed decrease in the IFN dependent GR binding was concomitant with a decrease in the steady state levels of GR mRNA. These results suggest that the decrease in GR mRNA levels was at least partially accountable for the alteration in GR binding. However, the precise mechanism of IFN induced decrease in GR mRNA is not yet known at the present time, but might involve transcriptional and/or post-transcriptional regulation.

In contrast to the results presented here, Salkowski & Vogel (1992a) reported that IFN induced an increase in the number of GR in the murine macrophage cell line R4w264.7 and in primary peritoneal exudate macrophages from C3H mice. More recently, they further demonstrated that lipopolysaccharide could also induce an increase in GR number in murine macrophages (Salkowski & Vogel, 1992b). However, the molecular basis and the potential significance of IFN-induced changes in GR have not been studied in their systems. Interestingly, Kam *et al.* (1993) reported that IFN had no effect on GR binding in peripheral blood mononuclear cells. The exact reasons for such contrasting effects of IFN on GR are not clear at the present time. It is most probably due to the different cells used. These totally different effects of IFN on GR from cell to sell suggest that IFN might affect receptor function in a tissue-specific manner, or that the receptor in different cells may be regulated differently, depending upon its cellular context.

Cytokine regulation of the GR has been reported in some cellular systems. Sica *et al.*, (1990) showed that natural interferon-β, but not recombinant α-2b-interferon, increased GR number in a human promyelocytic cell line (HL-60) by 30% as early as 24 h after treatment. Human recombinant interleukin-1 (IL-1) and natural interleukin-6 (IL-6) were found to be capable of diminishing the GR binding in Reuber hepatoma cells (Hill *et al.*, 1988; Stith *et al.*, 1989). IL-1 and IL-6 were also found to inhibit the activity of phosphoenolpyruvate enzyme (Hill *et al.*, 1986, 1988, 1989), a glucocorticoid inducible enzyme that is the rate limiting enzyme in gluconeogenesis. It has been proposed that IL-1 and IL-6 may induce hypoglycemia *in vivo* by down-regulating GR expression, thereby interfering with glucocorticoid action. More recently, Kam *et al.* (1993) reported that peripheral blood mononuclear cells incubated for 48 h in the presence of combination IL-2 + IL-4 had GR with significantly reduced binding affinity associated with an increase in GR number. However, when cells were treated with IL-2 alone or IL-4 alone, no changes in GR were observed. Furthermore, the alteration in GR with IL-2 + IL-4 was associated with a functional change in T cell response to glucocorticoids.

The original observations of this study that IFN alone did not affect the proliferation of HOS-8603 cells, but significantly attenuated the Dex-induced suppression of HOS-8603 cell proliferation suggest a possible direct relationship between GR number and glucocorticoid biological response. To elucidate further the subsequent biological significance of down-regulation induced by IFN, AKP activity in HOS-8603 cells in response to Dex alone or in combination with IFN was determined since the expression of AKP activity is well characterized to be induced by glucocorticoids in these cells (Song, 1994). As our early findings, treatment of HOS-8603 cells with Dex alone could lead to a significant increase in AKP activity in a time-dependent manner. In contrast, IFN had no detectable effect on AKP activity in these cells. However, the induction of AKP activity by Dex is attenuated when HOS-8603 cells were treated with Dex and IFN simultaneously. These results support the notion that cellular GR concentration is a limiting factor in glucocorticoid responsiveness of target cells. The decrease in GR level induced by IFN and thereby the response to glucocorticoids may have pathological and/or physiological significance.

GR is a hormone dependent transcriptional factor, which is a major determinant of glucocorticoid responsiveness. Therefore, it is of crucial importance to understand the factors that regulate GR expression. It has been shown that location in the cell cycle (Cidlowski & Michaels, 1977), state of development (Kilinyak *et al.*, 1989), aging (Chang & Roth, 1979), stress (Song *et al.*, 1991; Song, 1991) and endocrine status all influence the number of receptors (Cidlowski & Cidlowski, 1981; Svec & Rudis, 1981). The most intensively studied modulators are the glucocorticoid hormones. In most cases, glucocorticoids promote a reduction in GR levels by a process termed homologous down-regulation, which usually results in a corresponding decrease in glucocorticoid sensitivity. In addition the homologous regulation, accumulated data demonstrate that the GR levels could also be modulated by some factors other than glucocorticoids. Levels of cAMP were first shown to influence GR expression. Later on, it was reported that phorbol esters could also significantly increase both GR mRNA and glucorticoid binding site number (Hirai *et al.*, 1985; Domin *et al.*, 1991). More recently, we and others found that retinoic acid was capable of increasing GR concentrations in several cell lines (Reiss *et al.*, 1988; Song & Cheng, 1993). These wide spread findings of the heterologous regulations of GR may represent a common mechanism ensuring a functional interaction of glucocorticoids with modulating factors.

Materials and methods

Materials

RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. Ltd., Tokyo, Japan. [1,2,4 ^{3}H] dexamethasone (Dex) (47 Ci/mmol) was purchased from New England Nuclear, Boston, MA. [α-^{32}P] dCTP(>3000 Ci/mmol) was Amersham Corp. products. Dex was from Sigma Chemical Co. (St. Louis, MO). Recombinant human IFN with a specific activity of 2×10^7 IU/mg protein, was from Molecular Genetics, Second Military Medical University, Shanghai. DCC-FCS was prepared by treating fetal calf serum (FCS) with dextran-coated charcoal (DCC) to remove endogenous steroids. Nitrocellulose was obtained from Schleicher & Schuell, Inc. (Keene, NH). Random primed DNA labeling kit was purchased from Boehringer (Mannheim, Germany). The human GR cDNA probe was a gift from Dr Weinberger (Hollenberg *et al.*, 1985). All other materials used for cell culture and biochemical analysis were of the highest quality commerically available.

Cells and cell culture

The cell line used throughout the experiments was established from a surgical specimen of osteosarcoma (Lu *et al.*, 1990; song 1994). The culture conditions have been described previously (Song, 1993; Song *et al.*, 1993). Briefly, the cells were grown in RPMI 1640 medium, supplemented with antibiotics and 5% FCS or DCC-FCS at 37°C in a humidified atmosphere of 5% CO_2 and air. The monolayer cultures were routinely subcultured using a 0.05% trypsin-0.02% EDTA solution.

Growth experiments

In experiments to test the effect of Dex and IFN on cell proliferation, HOS-8603 cells were plated at a density of 2×10^4 cells/well onto 24-well clusters in medium supplemented with 5% DCC-FCS. 24 h after plating, the culture medium was replaced with medium containing increasing concentrations of Dex or Dex + IFN, or vehicle alone. After

4 days of culture, cells were harvested and enumerated by trypan blue exclusion. The results were expressed as a percentage of control.

The effect of IFN on glucocorticoid-induced growth suppression was further examined by means of colony assay (Song *et al.*, 1993). Briefly, cells were suspended in RPMI 1640 containing 0.8% methylcellulose, 15% FCS, and aliquots of the cell suspension were plated onto 96-well plate at a density of 500 cells/well in the absence or presence of 10 nM Dex, 10 IU/ml IFN or in the simultaneous presence of Dex and IFN. After 12 days of culture, the number of colonies was counted with an inverted microscope. A colony was defined as a cell cluster containing more than 30 cells.

Measurement of [^{3}H]Dex binding

Binding of [^{3}H] Dex to specific receptors was measured by a whole cell binding assay as described (Song & Cheng, 1993; Song, 1994). A single cell suspension of HOS-8603 cells was prepared from confluent monolayers. Cells were plated onto 24-well plates at a concentration of 4×10^5 cells/well in the absence or presence of IFN at the indicated concentrations and time interval, 48 h after the HOS-8603 cells were initially plated. The tissue culture medium was replaced with sérum-free RPMI containing increasing concentrations of [^{3}H] Dex (1.25–35 nM) in the absence or presence of 1000-fold excess unlabeled Dex. After incubation for 90 min (37°C, 5% CO_2), monolayers were washed over a 10 min time interval with four changes of cold phosphate-buffered saline. Cells were lysed in 0.5% deoxycholate for 30 min. Cell lysates were transferred to vials and radioactivity was measured in a scintillation counter. Cell number was assessed from two additional wells/treatment that were processed in a manner identical to that of the wells used in the whole cell binding assay. Non-specific binding was subtracted from total binding to determine specifically bound [^{3}H] Dex. The binding of [^{3}H] Dex to cells was expressed as the fmol of GR per millions cells. The dissociation constant (K_d) and maximal binding capacity (B_{max}) of [^{3}H] Dex to the receptor were determined by Scatchard analysis.

For whole cell, one-point binding assays which were performed as described above, a saturating concentration of [^{3}H] Dex (25 nM) was used in the absence or presence of 1000-fold excess radionert Dex. With this procedure, a strong correlation between one-point binding assay results and the maximal binding capacity calculated from Scatchard analysis of multipoint binding assays was consistently observed.

RNA blot analysis

Cells were lysed directly in the flasks in 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M β-mercaptoethanol, and the DNA sheared by homogenization. RNA was isolated as described by Chromcyzynski & Sacchi (1987). The extracted RNA was precipitated with 2.5 volume of ethanol and 0.1 volume of 3 M sodium acetate, quantitated by determination of the absorbance of a diluted aliquot at 260 and 280 nm. For dot blot hybridization analysis, total RNA was denatured with formaldehyde, diluted with 20 × SSC, applied onto nictrocellulose membranes using a filtration apparatus, and fixed by baking for 2 h at 80°C in a vacuum oven. The filters were hybridized with the ^{32}P-labeled 746 bp EcoRI fragment of human GR cDNA as described before (Song *et al.*, 1991). Rehybridization was performed with β-actin cDNA to verify that equal amounts of RNA had been loaded. The probes were labeled with [α-^{32}P]dCTP according to the random primer method and the quantification of mRNA was carried out by densitometric scanning of autoradiographs with a shimadzu dual-wavelength TLC scanner cs930 (Tyoko, Japan) and by integrating the peak areas.

Measurement of AKP activity

AKP activity was measured exactly as described before (Song *et al.*, 1993). The cells were washed with phosphate-buffered saline, trypsinized, and transferred into Eppendorf tubes. After centrifugation, cell pellets were suspended in 0.25 M sucrose and homogenized with a Teflon homogenizer. The homogenates were centrifuged at 3000 *g* for 5 min at 4°C. AKP activity in the supernatants was determined by measuring the release of phenol from disodium phenyl phosphate spectrophotometrically (520 nm) at 37°C, expressed as the generation of phenol nmol/min/mg protein. Total protein was measured with Lowry's method (Lowry *et al.*, 1951).

Statistical analysis

All data were expressed as mean ± SD of at least three different experiments. Statistical analysis was performed by using the Student's *t*-test.

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