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Role of glucocorticoid on interleukin-6-induced cellular functions in the mouse macrophage cell line (Mm 1)

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Received 16 December 1996; revised 23 January 1997; accepted 23 January 1997

Abstract

To discover a role of glucocorticoid on interleukin-6 (IL-6)-induced responses of a macrophage, we investigated the effect of IL-6 and/or dexamethasone (Dex) on cellular functions of a mouse macrophage cell line (Mm1 cells). The results obtained were as follows. (1) Dex decreased the accumulation of tumor necrosis factor-α induced by IL-6, whereas nitric oxide production was enhanced by Dex. Moreover, the enhancement of nitric oxide production could be demonstrated to be associated with stimulation of iNOS mRNA expression by the Dex treatment. (2) Cytotoxic activity of Mm1 cells on mouse B16 melanoma cells was much more enhanced by the co-treatment of IL-6 with Dex than IL-6 treatment alone. (3) Dex promoted further the suppression of proliferation induced by IL-6. (4) DNA fragmentation, introduced by the treatment of cells with IL-6, was further enhanced in the presence of Dex.

Keywords: TNF-α production; NO production; Cytotoxicity; Apoptosis; IL-6; Dexamethasone; Macrophage

1. Introduction

Glucocorticoids are well known as anti-inflammatory factors, which exhibit anti-inflammatory effects by stimulating or inhibiting the transcription of a variety of genes [1]. In macrophages, it has been reported that glucocorticoid hormones inhibit a variety of macrophage functions [2], especially recognized by the production of inflammatory mediators such as nitric oxide (NO) [3] and tumor necrosis factor-α (TNF-α) [4]. However, it is also the fact that glucocorticoids exert complicated actions from the view of anti-inflammation. The long-term administration of glucocorticoids results in serious adverse effects. In osteoporosis, dexamethasone (Dex) stimulates the osteoclast-like cell formation from mouse bone marrow culture systems [5], and the activation of osteoclasts [6]. In rat and human hepatic cells, glucocorticoids synergize with IL-6 to stimulate the production of acute-phase proteins [7,8].

Interleukin-6 (IL-6), originally identified as a B cell differentiation factor [9], has a variety of biologic functions. IL-6 acts on myeloma cells as a growth factor [10] and on hematopoietic stem cells as a multi-colony stimulating factor [11]. IL-6 induces cytotoxic T cells from murine thymocytes in the presence of IL-2 [12]. In addition, IL-6 also induces the differentiation of human B lymphocyte synergistically with glucocorticoids [13]. These diverse functions of IL-6 strongly implicate a role in the regulation of growth and differentiation of many cell types.

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PII S0167-4889(97)00020-7

However, many things to be clarified still have been remained in the function of IL-6, particularly in relation to Dex action.

In a mouse myeloid leukemia cells (M1 cells), both IL-6 [14] and Dex [15] act as differentiation factor. In our previous paper, production of NO in mouse macrophage cell line (Mm1), a subclone of M1 cells was reported to be markedly induced by IL-6 treatment [16]. In subsequent research of NO production from Mm1 cells by IL-6 treatment, we recognized that Dex does not suppress the NO production but rather enhances. Moreover, Dex was suggested to influence on another responses such as proliferation and cell death induced by IL-6 treatment. In this paper, we investigated about the diverse function of Dex on IL-6 actions using Mm1 cells.

2. Materials and methods

2.1. Materials

Dulbecco modified MEM culture medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). Recombinant human IL-6 was a gift from Dr. K. Yasukawa (Tosoh Niotechnology Research Laboratory, Ayase, Japan). Glucocorticoids and other chemical agents were purchased from Wako Pure Chemicals (Osaka, Japan). Culture dishes and micro well plate were obtained from Sumitomo Bakelite (Tokyo, Japan).

2.2. Cell lines and culture

M1 cell line was isolated from myeloid leukemic cells of an SL strain mouse [17]. Mm1 subclone was isolated from M1 and characterized in 1973 [18]. Mm1 cells were cultured in MEM culture medium containing 5% horse serum and two-fold concentration of amino acids plus vitamins.

2.3. Measurement of nitrite concentration for assessment of NO production

Nitrite concentration in the culture supernatant was measured by Griess reaction. Culture supernatant (50 μl) was mixed with 75 μl of 0.2 M acetate buffer and 75 μl of Griess reagent (containing 0.05% naphthyl-ethylene diamine, 0.5% sulfanilamide and 2.5% phosphorous acid). Absorbance was measured at 540 nm after incubated for 20 min at room temperature. Nitrite concentration was determined from a standard curve by using 0–60 nmol/ml of sodium nitrite dissolved in 5% HS-MEM.

2.4. RT-PCR analysis of NO synthase mRNA expression

Total RNA was prepared by AGPC method [19]. The final preparation was quantitated spectrophotometrically and then incubated with reverse transcriptase at 37 for 30 min. RT-PCR method was carried out according to the method described by Svetic et al. [20]. Oligonucleotide primers for NO synthase were synthesized based on the sequences described by Lyons et al. [21]. These primers (5′-primer: ACCTACTTCCTGGACATTACGACCC; 3′-primer: AAGGGAGCAATGCCCGTACCAGGCC) generated a 456 bp band. Oligonucleotide primers for β-actin (5′-primer: GTGGGCCGCTCTAGGCACCAA; 3′-primer: CTCTTTGATGTCACGCACGATTTC) were used as control. These primers generated a 540 bp band. PCR products were visualized by electrophoresis through agarose gel containing 1 μg/ml of ethidium bromide.

2.5. Assay for TNF activity

Mm1 cells (3×10^6 ceils) were seeded in a 35 mm diameter dish with 100 U/ml of IL-6 and/or 10^{-6} M of Dex. In the experiment using Dex, Mm1 cells were pre-treated for 24 h by 10^{-6} M of Dex, then the cell number was counted and seeded with 10^{-6} M of Dex in the presence or absence of IL-6 (100 U/ml). Basal TNF-α activity was determined by the incubation of Mm1 cells for 5 h (without stimulants). After incubation for the indicated times, the supernatants were collected and transferred to the L929 cells microplate assay system described as follows.

TNF activity monitored by the cell lytic assay essentially as described by Ruff and Gifford [22]. Briefly, mouse L929 fibroblastic cells were plated in 96-well plates at 30000 cells/0.1 ml to establish a dense monolayer. After incubation for 5 h at 37 in a humidified 5% CO_2 atmosphere, 50 μl of actinomycin D solution (4 μg/ml) and 50 μl of test

samples were added to the wells, and then the cells were further incubated for 18 h. At the end of the culture period, the supernatants were removed, washed twice with phosphate buffered saline, stained with glutaraldehyde (0.25%), and dyed with crystal violet (0.2% in 2% methanol) for 10 min. After the cells were washed with water and allowed to dry, the cells were solubilized with 1% SDS. The concentration of the dye was determined by measuring the absorbance at 546 nm. A dose-response standard curve was drawn using rMuTNF-α (Genzyme: TNF-M). The final concentrations of transferred rIL-6, dexamethasone alone or together did not influenced on the TNF-α-induced cytolysis of L929 cells in the presence of actinomycin D.

2.6. Cytotoxicity assay

Cytotoxicity was assayed by the method [23] counting ^{51}Cr released from labeled target cells as follows: Mm1 cells (2×10^5 cells/well) were pre-incubated for 24 h with various concentrations of IL-6 in the presence or absence of Dex (10^{-6} M) in a 48-well plate. After pre-incubation, ^{51}Cr-labeled mouse B16 melanoma cells (1×10^5 cells/well) were added and then incubated for more 3 days. After incubation, the radioactivity in the culture supernatant was counted by gamma-counter. For spontaneous release, the supernatant of target cells alone was counted. Moreover, cytolysis of ^{51}Cr-labeled B16 melanoma cells in this assay system was not induced by treatment of IL-6 and/or Dex. The radio activity in the supernatant of B16 cells treated with IL-6 (100 U/ml) and/or Dex (10^{-6} M) was not changed as compared to the none-treated cells. Cytotoxicity (%) was calculated as follows: [sample cells (cpm)spontaneous (cpm)]/[total incorporated radioactivity (cpm)spontaneous (cpm)]100 = cytotoxicity (%). To determine the total incorporated radioactivity (cpm), ^{51}Cr-labeled B16 melanoma cells (1×10^5 cells) without treatments of IL-6 and/or Dex were lysed completely with 1% Triton X-100 and the radio activity was counted.

2.7. [^{3}H]Thymidine incorporation

Mm1 cells (1.5×10^5 cells/well) were cultured for 2 days with treatment of IL-6 (100 U/ml) in the presence or absence of Dex (10^{-6} M) in a 48-well plate. Then, [^{3}H]thymidine (2 μCi, specific activity 40–60 Ci/mmol) was added to each well. After 4 h, cells were harvested on a glass fiber filter by washing with saline and 1% trichloroacetic acid and counted with scintillator by a liquid scintillation counter.

2.8. Culture conditions and DNA extraction for assessment of DNA fragmentation

Mm1 cells (1×10^7 cells/well) were cultured with treatment of IL-6 (100 U/ml) in the presence or absence of Dex (10^{-6} M) in a 90 mm diameter dish. After 3 days, cells were harvested by centrifugation and a portion of cells was utilized for DNA extraction. Another portion of cells was cultured continuously for three more days with fresh medium in the absence of inducers.

DNA was extracted according to the method described by Miller et al. [24]. Extracted DNA samples were then treated with Rnase for 30 min at 37 and reprecipitated with ethanol. DNA concentration was determined by measurement of absorbance at 260 nm. Electrophoresis was performed on a 1.2% agarose gel at 100 V for 30 min in the presence of 1 μg/ml ethidium bromide. A Hinc II digest of φX174 (am3cs70) (DNA MW Marker 5, Nippon Gene, Japan) was used as a standard.

2.9. Statistical analysis

The statistical significance of differences between the groups was determined by applying Student's *t*-test.

3. Results

3.1. Effect of glucocorticoids on NO production in il-6-induced Mm1 cells

The effect of glucocorticoids on NO production in IL-6-induced Mm1 cells was investigated (Table 1). These glucocorticoids possessed an enhancing effect on NO production to a greater or lesser extent. Dex showed the greatest effect among four glucocorticoids. The effect of Dex on NO production of Mm1 cells was further investigated in detail (Fig. 1). This

Table 1
Effect of glucocorticoids on NO production in IL-6-induced Mm1 cells

	Treatments of IL-6 and/or glucocorticoid (M)		NO production (nmol/10^6 cells)	Relative value
Control	None		4.5 ±90.99	
IL-6 (100 U/ml)	None		48.49 ± 3.45	1
	Dex	1×10^{-7}	70.38 ± 0.99 **	1.45
		1×10^{-5}	68.52 ± 2.73 **	1.41
	Cortisol	1×10^{-7}	62.54 ± 3.52 *	1.29
		1×10^{-5}	71.23 ± 1.28 **	1.47
	Corticosterone	1×10^{-7}	64.38 ± 5.77 *	1.33
		1×10^{-5}	49.34 ± 2.39	1.02
	Cortisone	1×10^{-7}	42.15 ± 3.30	0.87
		1×10^{-5}	56.00 ± 5.59	1.18

Mm1 cells were cultured for 72 h with IL-6 (100 U/ml) in the presence or absence of glucocorticoid. The NO production was then assayed using respective supernatants as described in Section 2.
The results are presented as means ± SD of quadruplicate. Significant difference from IL-6 treatment: * $P < 0.01$, ** $P < 0.005$.

enhancement was reached to maximum at 10^{-5}–10^{-7} M of Dex. Thus, Dex (10^{-6}M) was used in the following experiments. Moreover, it was found that production of NO was not induced by Dex treatment alone.

3.2. *Effect of Dex on expression of iNOS mRNA in il-6-induced Mm1 cells*

From the above results, iNOS mRNA expression was deduced to be stimulated by treatment of Dex to IL-6-induced Mm1 cells. Thus, effect of Dex on expression of iNOS mRNA was examined using IL-6-induced Mm 1 cells in the presence or absence of Dex (10^{-6}M) for 0–48 h (Fig. 2). By the treatment of Dex, expression of iNOS mRNA in IL-induced Mm1 cells was found to be remarkably stimulated prior to the increment of NO production. From the finding, the enhancement of NO production by Dex was demonstrated to be due to stimulation of iNOS mRNA expression, at least.

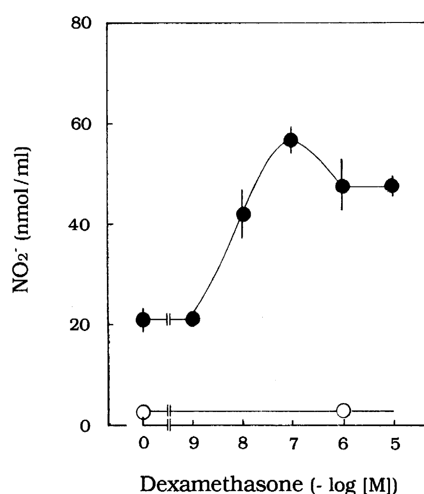

Fig. 1. Effect of Dex on NO_2^- production in IL-6-treated Mm1 cells. Mm1 cells were treated for 48 h by IL-6 (100 U/ml) in the presence (●) or absence (○) of Dex (10^{-9}–10^{-5} M). The culture supernatants were then assayed for NO_2^- concentration as described in Section 2. Results are expressed as means ± SD of four cultures.

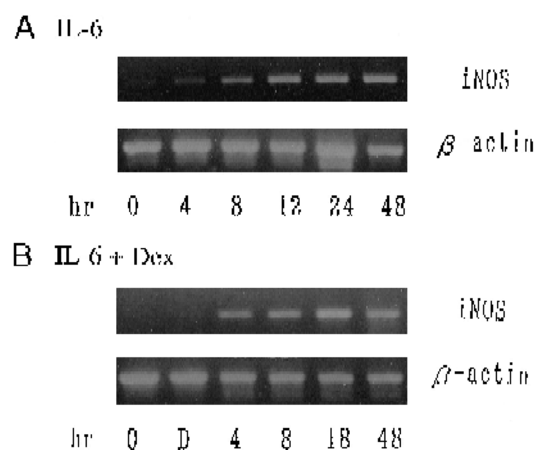

Fig. 2. Effect of Dex on iNOS mRNA expression in IL-6-induced Mm1 cells. Mm1 cells ($1.5\cdot10^5$ cells/cm^2) were incubated for the indicated times without (A) or with Dex (10^{-6} M) (B) in the presence of IL-6 (100 U/ml). Total RNA of each sample was reverse transcripted and amplified by using specific primers for iNOS and β-actin mRNA. PCR products were visualized by electrophoresis. D: Treatment of Dex alone (48 h).

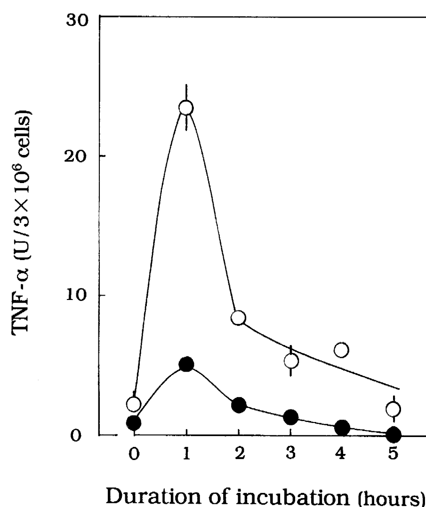

Fig. 3. TNF-α activities in the supernatants of Mm1 cells. Mm1 cells ($3 \cdot 10^3$ cells/35 mm diameter dish) were treated by IL-6 (100 U/ml) in the presence (●) or absence (○) of Dex (10^{-6} M). At the indicated times, TNF-α activities in the supernatant was measured as described in Section 2. Values are the means ± SD of four cultures.

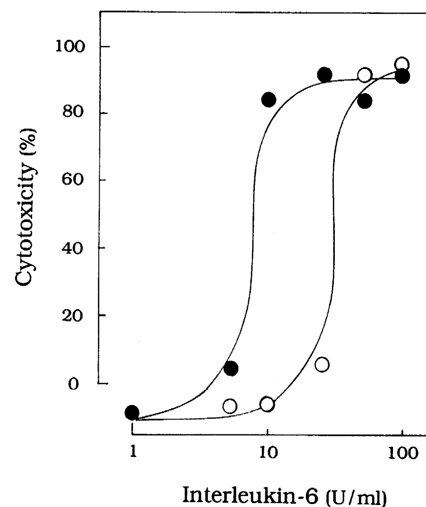

Fig. 4. Effect of Dex on IL-6-induced cytotoxic activity in Mm1 cells. Mm1 cells ($2 \cdot 10^5$ cells/well) were pre-incubated for 24 h by various concentrations of IL-6 in the presence (●) or absence (○) of Dex (10^{-6} M). After the incubation, ^{51}Cr-labeled mouse B16 melanoma cells ($1 \cdot 10^5$ cells/well) were added to the incubation mixture and then furthermore incubated. The cytotoxicity was calculated as described in Section 2. Results are expressed as means ± SD of four cultures.

3.3. Effect of Dex on TNF-α production in il-6-induced Mm1 cells

A little TNF-α activity without stimulant (open circles at 0 h) was detected in the supernatant of Mm1 cells during 5 h of the culture period as shown in Fig. 3. TNF-α activity in the supernatant of IL-6-treated Mm1 cells showed a maximum increase at 1 h after culture, and then gradually restored to control level for 4 h. Such an increment of TNF-α induced by IL-6-treatment was significantly suppressed by Dex as shown in Fig. 3. Moreover, the effect of IL-6 and/or Dex on TNF-α-induced cytolysis in actinomycin D-treated L929 cells was examined with various concentrations of TNF-α by incubations of IL-6-treated Mm1 cells, which were cultured in the presence or absence of Dex. However, the TNF-α-induced cytolysis was not changed by the treatments of IL-6 and/or Dex (not shown data).

3.4. Effect of Dex on cytotoxic activity of Mm1 cells versus tumor cells

The cytotoxicity of Mm1 cells versus tumor cells was examined using mouse B16 melanoma cells as shown in Fig. 4. Mm1 cells acquired dose-dependently the cytotoxic activity by the treatment of IL-6.

Table 2
Effect of IL-6 and/or Dex on [^{3}H]thymidine in incorporation in Mm1 cells

Treatment	[^{3}H]TdR incorporation (dpm/culture)	Relative value
Control	521 547 ± 43 318	1
Dex ($1 \cdot 10^{-6}$ M)	378 337 ± 40 724 *	0.73
IL-6 (100 U/ml)	194 314 ± 31 888 *	0.37
IL-6 (100 U/ml)Dex ($1 \cdot 10^{-6}$ M)	15 772 ± 3339 *	0.03

Mm1 cells were cultured for 48 h with IL-6 and/or Dex. [^{3}H]Thymidine (TdR) incorporation during the final 4 h in culture was measured as described in Section 2.
The results are expressed as mean ± SD of quadruplicate.
* Significant difference from control: $P < 0.005$.

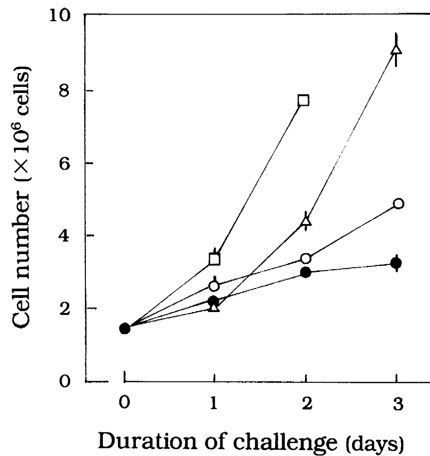

Fig. 5. Changes in cell number after the challenge of rIL-6 and/or Dex treatment. Mm1 cells were incubated in 60 mm diameter dish at 1.5×10^{6} cells with medium alone (□), 10^{-6} M of Dex (△), 100 U/ml of rIL-6 (○), or with both (●) Dex (10^{-6} M) and rIL-6 (100 U/ml). After incubation for the indicated times, cells were harvested and counted. Results are expressed as means ± SD of four measurements.

B16 melanoma cells were introduced to lysis of more than 90% by the co-culture with the IL-6 (50 U/ml)-treated Mm1 cells. Moreover, the cytotoxic activity of IL-6- treated Mm1 cells was markedly stimulated by Dex; nearly 90% of B16 cells were lyzed by co-culture of the Mm1 cells, which were treated with a smaller amount (10 U/ml) of IL-6 in the presence of Dex (10^{-6} M).

3.5. Effect of Dex on proliferation of Mm1 cells

The effect of Dex on proliferation of Mm1 cells was examined by the measurement of [^{3}H]thymidine incorporation at day 2 (Table 2) and by the changes of cell number (Fig. 5). The [^{3}H]thymidine incorporation was suppressed by the challenge of IL-6 treatment, but a portion of cells still proliferated. Co-treatment of Dex with IL-6 suppressed the [^{3}H]thymidine incorporation almost completely. These results were consisted with the changes of cell number. [^{3}H]Thymidine incorporation was slightly suppressed by the treatment of Dex alone. The suppression might be due to decrease of the cell numbers as compared to control.

3.6. Effect of Dex on the induction of apoptotic cell death

The molecular alterations of DNA under the various conditions after treatments of 10^{-6} M Dex and/or 100 U/ml of IL-6 are shown in Fig. 6. At the ends of 3 days culture, DNA fragmentation proceeded slightly in cells treated with Dex alone. This fragmentation might be induced by apoptosis within first day so that the number of Dex-treated cells was scarcely increased from 0 to 1 day (Fig. 6). IL-6 was suppressive to this Dex-induced DNA fragmentation. On the other hand, IL-6-treated Mm1 cells were undergoing DNA fragmentation when cultured subse-

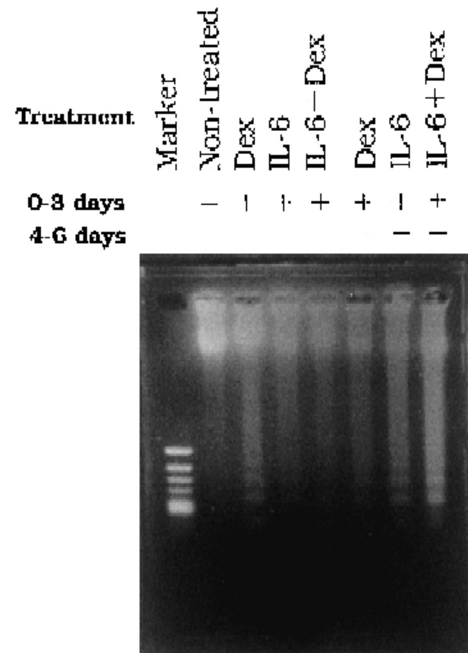

Fig. 6. Effect of Dex on the induction of DNA fragmentation in IL-6-induced Mm1 cells. DNA was extracted from Mm1 cells as described in Section 2, and 2 μg DNA was run on agarose gels. A standard 79 to 1,057-bp DNA is shown in lane 1. Lane 2 corresponds to DNA extracted from non activated Mm1 cells. Other lanes correspond to DNA harvested from Mm1 cells at the ends of cell culture in the presence or absence of Dex (10^{-6} M) and/or IL-6 (100 U/ml). Dex (lane 3), IL-6 (lane 4) and Dex + IL-6 (lane 5) were cultured for 3 days (0–3 day) in the presence of their stimulants. Dex (lane 6), IL-6 (lane 7) and Dex + IL-6 (lane 8) were cultured for 3 days in the same conditions and then furthermore incubated for 3 days (4–6 day) after removals of the stimulants.

quently after the withdrawal of IL-6. This DNA fragmentation was more remarkable in the Mm1 cells which were treated with IL-6 in the presence of Dex.

4. Discussion

In this study, we first examined the effect of glucocorticoids on NO production. Dex and the other glucocorticoids were found to enhance the NO production induced by IL-6 treatment. Moreover, iNOS mRNA expression in IL-6-induced Mm1 cells was demonstrated to be stimulated prior to the enhancement of the NO production by Dex treatment. The reports so far have been described that the effect of Dex on NO production is only suppressive [25]. Nonetheless, it was also confirmed by another macrophage cell line that Dex preferably enhanced the NO production induced by IFN-γ (manuscript in preparation). From their evidence, effect of Dex on NO production may vary by the cell types.

On the other hand, it has not been clarified how can Dex affect the expression of iNOS mRNA. The promoter/enhancer region of murine iNOS gene has been cloned, and the elements that several transcriptional factor can bind have been identified [26,27]. However, the iNOS promoter/enhancer region lacked the steroid response elements. An element that activator protein-1 can bind existed in this region [26,27], and it was reported that glucocorticoid receptor associates with activator protein-1 directly to regulate the transcriptional control [28]. However, the regulation system is now questioned in terms of direct association.

As NO has been demonstrated to be a mediator of inflammation, the enhancement of NO production by Dex may be inconsistent with the fact. Nonetheless, a dramatic increase in hepatic damage by blocking the NO production has been demonstrated in inflammation-inducing mice model [29]. This result suggests that NO is capable of exhibiting the anti-inflammatory action under the specific circumstance. Furthermore, it is noteworthy that hepatocytes produce acute-phase proteins in response to IL-6, and Dex synergistically enhances the proteins through the interaction of IL-6-response elements [7,8].

Dex which lowered the concentrations of IL-6 to obtain the same degree of cytolytic activity, may be due to the enhancement of NO production. Thus, there was no effect of TNF-α production on this assay, so that B16 melanoma cells were co-cultured with Mm1 cells pre-treated by IL-6 and/or Dex for 24 h. TNF-α has been reported to have an enhancing effect of NO production [30]. Indeed, TNF response element exists in the promoter/enhancer region on iNOS gens [26,27]. However, endogenous TNF-α did not influence on the NO production in IL-6-treated Mm1 cells, because only high concentrations of recombinant TNF-α was rather suppressive for the NO production and the neutralizing antibody for TNF-α had no effect (data not shown).

On the other hand, the endogenous NO and TNF-α might be able to influence on the change of proliferative ability and the induction of cell death. As for the influence of NO on the proliferation, our laboratory had reported that endogenous NO participated in the blocking the cell cycle of IL-6-induced Mm1 cells in the early $G_2 + M$ phase [31]. It is expected that this tendency is more emphasized under the condition of increased NO production by Dex co-treatment, On the other hand, TNF-α do not influence on the proliferation of Mm1 cells [32].

Some reports insist on the correlation of produced NO with apoptosis in activated macrophages [33,34]. In view of the mechanism of apoptosis induced by NO, it might be mediated by the inhibition of mitochondrial Fe-S containing enzymes [35], since the inhibitor of mitochondrial electron flow was shown to be able to induce apoptosis [36]. In addition, structural damage of mitochondria parallel to NO production in host cells had been recognized [37]. A portion of our results supports this notion, because the degree of DNA fragmentation subsequent to the activation was more proceeded in relation to the enhancement of NO production by the co-treatment of Dex with IL-6. However, these mechanism, namely the trigger of DNA fragmentation by NO, will not account for that of Mm1 cells entirely. Since these signal should be transmitted soon and DNA fragmentation should be resulted in a relatively short time, whereas DNA fragmentation in Mm1 cells induced by IL-6 with/without Dex gradually progressed after the removal of stimulus when the series of NO production had been completed. Moreover, Dex alone treatment induced the DNA fragmentation to some

extent in a relatively early stage despite the no NO production.

As for the Dex-induced apoptosis, it has been shown that Dex induces thymocyte apoptosis accompanied with DNA fragmentation caused by the activation of calcium-dependent endonuclease [38,39]. So the DNA fragmentation induced by Dex alone treatment in Mm1 cells might be introduced by the same mechanism as above. It is notable that IL-6 was inhibiting to this Dex action.

In contrast, the DNA fragmentation induced by IL-6 treatment was resemble to the activation-induced apoptosis that is introduced in the terminal differentiated macrophages as Munn and co-workers [40] demonstrated. Dex might proceeded this programmed cell death by acting as an enhancer of differentiation and activation in association with IL-6. This system can be evaluated as anti-inflammatory effect of Dex; it may cause the serious situation for host body if activated-macrophages remain at the site of inflammation.

In this study, we demonstrated that glucocorticoids are an important role for IL-6-induced cellular functions in Mm1 cells using Dex.

Acknowledgements

The authors gratefully acknowledge the kind suggestions of Dr. K. Takagi (in our department) for this study.

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