A glucocorticoid, dexamethasone, inhibited the production of a leukocyte chemotactic cytokine, interleukin 8 (IL-8), as well as mRNA expression by a glioblastoma cell line, T98G, stimulated with interleukin 1 (IL-1). Dexamethasone also inhibited IL-8 promoter-driven chloramphenicol acetyltransferase (CAT) activities induced by IL-1, suggesting that dexamethasone inhibited IL-8 production mainly at the transcriptional level. Moreover, CAT assay revealed that the nuclear factor-kB (NF-kB) binding site was the crucial cis-element required for conferring IL-1 responsiveness in conjunction with the CCAAT enhancer binding protein/nuclear factor-IL-6 (NF-IL6) and/or the AP-1 binding site(s). Mutation of either the AP-1 or NF-IL6 binding site did not abolish IL-8 gene repression by dexamethasone, suggesting that these sites were not targets for dexamethasone. Trimerized kB sequence in the IL-8 gene was enough for conferring the induction by IL-1 and inhibition by dexamethasone of CAT activity. Finally, dexamethasone diminished the IL1-induced formation of NF-kB complexes, which were identified immunochemically to consist of p50 and p65, without reducing the amount of translocated factors. Collectively, dexamethasone interfered with the binding of the most essential transcription factor, NF-kB, to its cognate cis-element, thereby suppressing the transcription of IL-8 gene.

The early phase of inflammation consists of plasma leakage and infiltration of leukocytes, particularly neutrophils into the lesion. Infiltrated neutrophils generally play a protective role by eradicating the cause of inflammation (1). However, if excessive numbers of neutrophils migrate into the lesion, neutrophils induce tissue damage by releasing their lysosomal enzymes or generating superoxide anions. Thus, close regulation of neutrophil migration is necessary to control inflammatory reactions.

Neutrophils migrate according to the concentration gradient of chemotactic factor(s) (2). A novel polypeptide leukocyte chemotactic factor, interleukin 8 (IL-8),1 exhibits in vitro chemotactic activity for T cells and basophils as well as neutrophils (3, 4), affects the adhesion of neutrophils to the endothelium (5), and induces the trans-endothelial migration of neutrophils (6). The intradermal administration of IL-8 induces infiltration of neutrophils and T cells (7). We observed that the administration of large amounts of IL-8 into rabbit knee joints caused the destruction of synovium in a neutrophil-dependent manner (8). Furthermore, the administration of a neutralizing antibody against IL-8 inhibited neutrophil infiltration and tissue damage in several types of acute inflammation (9, 10), suggesting a causal role of IL-8 in inflammatory reactions.

Various types of inflammatory stimuli including lipopolysaccharide, IL-1, and tumor necrosis factor a induce IL-8 production at the transcriptional level in a wide variety of cells (3, 4). We observed that IL-8 transcription required either the combination of NF-kB and C/EBP/NF-IL6 or that of NF-kB and AP-1 binding sites in the promoter region of the IL-8 gene, depending on the types of cells (11-13). The NF-kB binding site is indispensable for IL-8 gene expression in all the cell types that we have examined.

Excessive production of IL-8 may be involved in the pathogenesis of several types of inflammatory reactions, particularly neutrophil-dependent tissue damages. Thus, the suppression of IL-8 production may be beneficial for the control of various types of inflammatory reactions. Several cytokines and agents have been reported to inhibit IL-8 production. Among these, IL-4 (14), 1,25-(OH)2-vitamin D3 (15), cyclosporin A (16), interferon (17), and glucocorticoids (18, 19) have been demonstrated to suppress IL-8 mRNA expression in several types of cells, although the molecular mechanism of IL-8 gene repression remains to be investigated.

In this study, we analyzed the molecular mechanism of IL-8 gene repression by dexamethasone in a human glioblastoma-derived cell line, T98G. We observed that only mutation of the NF-kB binding site abolished IL-8 gene repression by dexamethasone and IL-8 gene activation by IL-1. Furthermore, dexamethasone decreased IL-1-induced NF-kB complexes that were identified immunochemically to consist of p65 and p50.

EXPERIMENTAL PROCEDURES

Cell Line—A human glioblastoma cell line, T98G, was obtained from the Japanese Cancer Research Resources Bank (Kamigyoba, Tokyo, Japan). The cells were maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5% fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin.

recombinant interleukin; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT enhancer binding protein; EMSA, electrophoretic mobility shift assay; bp, base pairs; AP-1, activator protein-1.
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Cytokines and Reagents—Human recombinant interleukin 1α (rIL-1α) and rIL-8 were generously provided by Dainippon Pharmaceutical (Osaka, Japan). Preparation methods of rabbit antisera against c-Rel have been described previously (20). Rabbit antisera against the peptides of the N terminus of human p65 (DELFLFLIPFAPAQAS), the N terminus of human p52 (ESCYNPGLDGIIEYDD) were raised as previously described (21). The specific antisera against NF-IL6 were a kind gift from Dr. Akira (Osaka University) (22). Dexamethasone (Sigma) was dissolved in ethanol at a concentration of 10 mg/mL and stored at −20 °C until use.

Measurement of IL-8 by Enzyme-linked Immunosorbent Assay—The content of IL-8 in the culture supernatants was determined by enzyme-linked immunosorbent assay as previously described (23) except that polyethylene glycol was excluded from the dilution buffer. The detection limit of this assay was consistently less than 20 pg/mL.

RNA Extraction and Northern Blotting Analysis—T98G cells were grown to subconfluency in tissue culture medium supplemented with 5% fetal bovine serum. Cells were stimulated by adding 10 ng/mL IL-1α in the presence or absence of dexamethasone at 37 °C for 3 h. Total RNA was extracted by single-step guanidine isothiocyanate centrifugation. Ten µg of total RNA was loaded and separated on a 1.0% agarose gel containing 5% formaldehyde and was blotted onto the GeneScreen (DuPont NEN). The membranes were baked, prehybridized, hybridized with 32P-labeled IL-8 cDNA (0.45-kilobase EcoRI-EcoRI coding region), and washed as previously described (24). Hybridization with human 18S ribosomal RNA confirmed that equal amounts of RNA were used for each blotting.

Transfection and CAT Assay—CAT expression vectors harboring the 5′-flanking region of IL-8 gene were constructed as described (11-13). The transfection of these plasmids into T98G cells was performed by the calcium phosphate co-precipitation method modified by Chen and Okayama (25). The CAT activity in cell extract was determined using an equal amount of proteins by the method described by Gorman et al. (26). After thin-layer chromatography, radioactivity was measured using a Bioimage Analyzer BAS2000 (Fuji-film, Tokyo, Japan), and percent conversion was calculated as counts/min in the acetylated form divided by total counts/min (12, 13). Slot blot analysis was performed using a constant aliquot of the same cell suspensions before incubation with [3H]thymidine (27). In some experiments, nuclear extracts were incubated with [3H]thymidine before hybridization with human 18S ribosomal RNA (24). Hybridization with human 18S ribosomal RNA confirmed that equal amounts of RNA were used for each blotting.

Requirement of NF-κB-like Binding Sites for Transcription of IL-8 Gene—Since we observed that the cis-elements indispensable for IL-8 gene transcription varied among different cell types, we investigated the cis-elements required for IL-8 gene transcription in T98G cells. CAT expression vectors were prepared by inserting the CAT gene linked to various types of deleted or mutated IL-8 promoters (28). Deletion below −98 bp caused little change of CAT activity induced by IL-1, indicating that the AP-1 site (from −126 to −120 bp) was dispensable for IL-8 gene expression induced by IL-1 in this cell line. This notion was further supported by the results of the transfection with three AP-1-mutated CAT vectors, all of which conferred the responsiveness to IL-1 (see Fig. 4B). However, the inducibility of CAT activity was greatly decreased when cells were transfected with −85-CAT (Fig. 2B). Since two cis-elements, C/EBP/NF-IL6 binding site (−94 to −81 bp) and NF-κB site (−80 to −70 bp), are located in the region (11), we examined whether these elements were involved in IL-8 gene activation. The inducibility of CAT activity was conferred to cells transfected with −94(Δ7−81)-CAT, which possesses both C/EBP/NF-IL6 and NF-κB sites (Fig. 3B). Mutation of −94(Δ7−81)-CAT at either the C/EBP/NF-IL6 (mutant A-CAT in Fig. 3) or the NF-κB site (mutant B-CAT in Fig. 3) abolished the induction of CAT activity upon stimulation with IL-1. The essential role of the NF-κB site has been further supported by the fact that the inducibility of CAT activity was lost when the NF-κB site of the −135-CAT was mutated (Fig. 4B). However, when cells were transfected with plasmid containing a mutated C/EBP/NF-IL6 and an intact AP-1 site (Fig. 4B), CAT activity was induced by the stimulation with IL-1. Collectively, the NF-κB binding site was involved in IL-8 gene activation in conjunction with C/EBP/NF-IL6 and/or AP-1 site(s) in this cell line.

IL-1-responsive Element Was Also Dexamethasone-sensitive Element—The deletion of the glucocorticoid-responsive element in the IL-8 gene had little, if any, effect on the IL-8 gene repression by dexamethasone in T98G cells (Fig. 2B). Several independent groups observed that a glucocorticoid affected the function of an essential transcriptional factor, AP-1 complex, thus leading to repression of a collagenase gene (29-32). In T98G cells, neither deletion nor mutation of a potential AP-1 binding site abolished the IL-8 gene repression by dexamethasone (Figs. 2B and 4C), suggesting that the AP-1 binding site was not essential for the gene repression by dexamethasone. The effects of mutation of the C/EBP/NF-IL6 binding site on gene repression varied, depending on the presence of an intact AP-1 binding site. Although IL-1-induced CAT activity was abolished by mutation of the C/EBP/NF-IL6 binding site in the absence of the AP-1 binding site (mutant A-CAT in Fig. 3), IL-8 gene repression by dexamethasone was not affected by mutation of the C/EBP/NF-IL6 site in the presence of the AP-1 site.
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Fig. 1. A, a human glioblastoma cell line, T98G, was grown in 60-mm plastic dishes in 2 ml of RPMI 1640 medium supplemented with 5% fetal bovine serum until reaching subconfluency. Cells were then stimulated with 10 ng/ml human rIL-1α in the presence of various concentrations of dexamethasone (DEX) for 24 h. IL-8 contents in supernatants were determined as described under "Experimental Procedures." B, T98G cells in a subconfluency state in 100-mm plastic dishes were stimulated with 10 ng/ml human rIL-1α in the presence of the indicated concentrations of dexamethasone for 3 h. Total RNA was then extracted and 10 μg of total RNA was loaded onto each lane. After transfer onto a nylon membrane, hybridization was performed as described under "Experimental Procedures." C, T98G cells were transfected with -546-CAT as described under "Experimental Procedures." After transfection, cells were stimulated with rIL-1α (10 ng/ml) in the presence of the indicated concentrations of dexamethasone for an additional 24 h. CAT activities in cytosol fractions were determined as described under "Experimental Procedures." Three experiments were repeated and representative results were shown.

Fig. 2. A, schematic structure of the 5′-flanking region in the IL-8 gene is shown. B, T98G cells were transfected with CAT expression vectors into which various 5′-deleted fragments were cloned. After transfection, cells were stimulated with rIL-1α (10 ng/ml) in the presence or absence of dexamethasone (DEX) (10⁻⁶ M), and after an additional 24 h intracellular CAT activities were determined as described under "Experimental Procedures." Dotted, open, and striped bars indicate intracellular CAT activities in cells stimulated with medium (med), IL-1α, and IL-1α plus dexamethasone, respectively.

Fig. 3. A, the 5′-flanking region of the IL-8 gene spanning from -94 to -70 bp is shown. The sequences of two point mutants, A and B, as well as -94 (d70-51)-CAT, are shown. B, the effects of point mutations on the inducibility of CAT activities by IL-1 are shown. The cells were transfected with the indicated CAT expression vectors. Intracellular CAT activities were determined on cells stimulated with IL-1α (10 ng/ml) in the presence or absence of dexamethasone (DEX) (10⁻⁶ M) for an additional 24 h. Dotted, open, and striped bars indicate intracellular CAT activities in cells stimulated with medium (med), IL-1α, and IL-1α plus dexamethasone, respectively.

activity (Figs. 3B and 4B), whereas the transfection with 3 x (IL-8-kB)-CAT and 2 x (AP-1-kB)-CAT, but not with 3 x (C/EBP/NF-IL6)-CAT and 2 x (AP-1)-CAT conferred the induction by IL-1 and the inhibition by dexamethasone of CAT activity (Fig. 5). These data raised the possibility of the NF-κB site as the
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**Fig. 5.** Trimerized IL-8-κB site (−80 to −71 bp) and C/EBP/NF-IL6 site (−94 to −81 bp) and dimerized AP-1 site (−126 to −120 bp) and Igκ-κB (5'-GGGACTTTCC-3') were synthesized and cloned into −50-CAT (11). Transfection was performed as described under "Experimental Procedures." Intracellular CAT activities were measured on cells stimulated for an additional 24 h with medium (med, dotted bars), IL-1α (10 ng/ml, open bars), and IL-1α (10 ng/ml) plus dexamethasone (DEX, 10−6 M, striped bars).

**Fig. 6.** EMSA using the C/EBP/NF-IL6 binding site as probe. Nuclear proteins were extracted from THP-1 cells stimulated for 1 h with medium (lane 1), IL-1α (10 ng/ml, lanes 2-5), or IL-1α (10 ng/ml) plus dexamethasone (10−6 M, lanes 6-9). EMSA was performed on nuclear extracts preincubated with no reagents (lanes 1, 2, and 6), C/EBP/NF-IL6 probe (lanes 3 and 7), anti-NF-IL6 (lanes 4 and 8), or anti-p65 (lanes 5 and 9).

IL-1 also induced the formation of NF-κB complexes that were inhibited by a specific oligomer but not by a mutated oligomer (Fig. 7). The NF-κB complexes were composed of p50-p65 heterodimers, as revealed by the facts that specific antisera against p50 or p65, but not those against c-Rel or p52, super-shifted the complexes in EMSA (Fig. 8). In addition, the NF-κB complexes were not affected by C/EBP/NF-IL6 or AP-1 oligomer, making it unlikely that the NF-κB complexes were physically associated with either NF-IL6 or AP-1 complex. Furthermore, dexamethasone diminished the formation of NF-κB complexes without changing their components (Fig. 7), whereas it failed to affect that of NF-IL6 (Fig. 6). Taking the results of CAT assays into consideration, these data suggested that NF-κB is the main target site of dexamethasone.

**Stimulation with medium IL-1**

**_complexes without changing their components (Fig. 7), whereas**

**NF-κB is the main target site of dexamethasone.**
**DISCUSSION**

Accumulating evidence indicates that IL-8 is essentially involved in one of the main features of inflammation, neutrophil infiltration, in several types of inflammatory reactions including lipopolysaccharide-induced acute dermatitis and ischemia-reperfusion injury of the lung (9, 10). Moreover, it has been reported that IL-8 could induce angiogenesis, a phenomenon closely related to inflammatory reactions (33). These findings suggest that aberrant production of IL-8 would have profound effects on the inflammatory reactions and that the production of IL-8 should be strictly controlled. This notion was further strengthened by the fact that most types of cells do not produce IL-8 constitutively but only in the presence of stimuli such as lipopolysaccharide, IL-1, and tumor necrosis factor (3, 4).

Our previous studies demonstrated that either the combination of NF-κB and C/EBP/NF-IL6 or that of NF-κB and AP-1 binding sites was the minimal essential cis-elements for IL-8 gene activation depending on the type of cells (11--13). In T98G cells, the combination of NF-κB and C/EBP/NF-IL6 binding sites was essential for IL-8 gene activation. However, IL-1-induced CAT activity was not ablated by the transfection with CAT expression vectors whose C/EBP/NF-IL6 binding site was mutated in the presence of an intact AP-1 binding site. In a human gastric cancer cell line, MKN45, in which NF-κB and AP-1 binding sites were the minimal enhancer regions required for IL-8 gene activation (13), no complexes were detected using C/EBP/NF-IL6 binding site as probe, implying the absence of trans-acting factor bound to this region. Collectively, the AP-1 site induces IL-8 gene transcription in concert with the NF-κB site only when the C/EBP/NF-IL6 binding site is mutated or when the corresponding trans-acting factor is absent in the cell.

EMSA revealed that IL-1 induced the formation of both NF-κB and C/EBP/NF-IL6 complexes. Moreover, the NF-κB complexes were supershifted by specific antisera against either p50 or p65 but not other members of the c-Rel family, while a specific antibody against NF-IL6 abolished the formation of both NF-κB and C/EBP/NF-IL6 complexes. We observed that co-transfection of p50, p65, and NF-IL6 expression vectors synergistically enhanced IL-8 promoter-driven CAT gene transcription (34). Collectively, IL-1 may induce the binding of p50-p65 heterodimers and NF-IL6 to the corresponding cis-elements, thus synergistically activating the IL-8 gene. Several independent groups demonstrated in vitro a direct physical association of the bZIP

region of C/EBPs including NF-IL6 with the rel domain of NF-κB proteins (35, 36), which was presumed to be responsible for synergism in gene expression by these transcription factors. However, we did not obtain any direct evidence of the physical association between these two factors since the addition of NF-IL6 probe or anti-NF-IL6 antibody had no effects on NF-κB complex formation. We could not completely rule out the possibility of a weak physical association of NF-κB with NF-IL6 that could not be detected by EMSA, and it remains to be determined how these two distinct types of transcription factors synergistically induced IL-8 gene transcription.

Glucocorticoids are used as immunomodulators and inhibit the gene transcription of several cytokines, particularly pro-inflammatory ones including IL-1 (37), tumor necrosis factor α (38), IL-8 (19), and monocyte chemotactic and activating factor (39). In T98G cells, the glucocorticoid response element contributed little, if any, to IL-8 gene repression by dexamethasone as the deletion of this element had negligible effects on gene repression. Several independent groups observed that a glucocorticoid inhibits collagenase gene transcription through interference with the function of AP-1 complexes without diminishing the binding of fos-jun to its cognate cis-element (40). In the collagenase gene, binding of fos-jun to the AP-1 binding site was the essential phenomenon leading to gene transcription (40). Glucocorticoid interfered with this essential machinery for gene transcription, thus suppressing the gene activation. However, in our system the AP-1 binding site was dispensable for IL-8 gene activation. Moreover, deletion or mutation of the AP-1 binding site failed to abolish IL-8-promoter-driven gene repression by dexamethasone, arguing against the assumption that the AP-1 binding site was the target site for dexamethasone treatment did not diminish the formation of NF-IL6 complex induced by IL-1. Although mutation of the NF-κB site completely decreased CAT activity induced by IL-1, both the induction by IL-1 and inhibition by dexamethasone were observed upon transfection with 3 × (NF-κB)-CAT, raising the possibility of this site as the responsible element for dexamethasone-induced repression. In addition, EMSA revealed that dexamethasone treatment significantly diminished the formation of NF-κB complex formation induced by IL-1. Since inhibitory actions of glucocorticoids are exerted on limited numbers of genes, it is unlikely that glucocorticoids repressed genes of inflammatory cytokines through acting on the basal machinery of transcription. Collectively, our results favor the assumption that the NF-κB site was responsible for IL-8 gene repression by dexamethasone.

Western blotting analysis on nuclear extracts demonstrated that IL-1 induced the translocation of both p50 and p65. Moreover, dexamethasone did not significantly diminish the amount of the translocated p50 and p65, suggesting that dexamethasone interfered with the binding of p50-p65 complex to NF-κB binding site. Direct binding of glucocorticoid receptor to DNA was presumed to displace the CAMP-responsive element binding factor that was essential for human glycoprotein hormone α-subunit, leading to the gene repression (41). A similar mechanism has been presumed to be involved in pro-opiomelanocortin gene repression by a glucocorticoid (42). However, we did not detect the appearance of a novel complex on EMSA after the treatment with dexamethasone, arguing against the possibility that a glucocorticoid receptor displaced NF-κB complexes. In the case of prolatin gene, a glucocorticoid repressed the gene transcription through the association of a hinge domain of glucocorticoid receptor with an essential transcription factor, Pit-1 (43). We could not immunohistochemically detect the glucocorticoid receptor in the NF-κB complex formed in the presence of IL-1 and dexamethasone. However, we cannot exclude the possibility that glucocorticoid-glucocorticoid receptor complexes have some effects on the binding of p50-p65 complexes to NF-κB element that are not detectable by EMSA.

It was reported that a glucocorticoid (44) as well as cyclosporin A and FK506 suppressed IL-2 gene activation through affecting NF-AT complexes. Recently, we observed that FK506 interfered with NF-κB complex formation in the IL-8 gene (46) that lacks the NF-AT binding site (18). Considering that several types of immunophilins could bind with glucocorticoid receptor as well as cyclosporin A and FK506, these two distinct kinds of immunosuppressants may suppress cytokine gene transcription through the NF-AT site in the case of IL-2 and the NF-κB site in the case of IL-8 gene. The elucidation of the mechanism of IL-8 gene repression by these agents may indicate new directions for the development of anti-inflammatory agents.

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References

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