Down-regulation of Interleukin 6 Receptors of Mouse Myelomonocytic Leukemic Cells by Leukemia Inhibitory Factor*

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We examined the effect of leukemia inhibitory factor (LIF) on the expression of interleukin 6 receptors (IL-6R) on mouse myelomonocytic leukemic M1 cells. Binding studies using 125I-labeled human and murine IL-6 revealed that LIF caused a decrease in IL-6 binding to M1 cells. The decrease became evident within 1 h, and the maximum decrease was observed at 3–6 h. Scatchard plot analysis revealed that M1 cells had a single class of high affinity receptors for IL-6 and that LIF-induced decrease in IL-6 binding was due to a decrease in the number of IL-6R on the cell surface and not to changes in their affinity. The affinity of IL-6R on M1 cells to human IL-6 ($K_d$ = 2.25 nM) was 10-fold lower than that to murine IL-6 ($K_d$ = 200 pM). The amount of IL-6 secreted into culture media by M1 cells that were treated with LIF for up to 12 h was not enough to cause receptor down-regulation. Northern blot analysis demonstrated that IL-6R mRNA was down-regulated by LIF treatment, and similar regulation was also observed when the cells were treated with IL-6. The time course of the IL-6R mRNA level was similar to that of IL-6R expression on the cell surface, suggesting that the main mechanism responsible for the loss of high affinity IL-6R was the regulation of IL-6R mRNA. Although the half-life of IL-6R on the cell surface was about 30 min, the addition of LIF reduced it to 16 min, suggesting the existence of an additional mechanism responsible for the loss of high affinity IL-6R on the cell surface.

Leukemia inhibitory factor (LIF) is a glycoprotein purified from conditioned medium of mouse fibroblast L929 cells (1) and Krebs II ascites cells (2) and induces a terminal differentiation of mouse myelomonocytic leukemic M1 cells into macrophage-like cells. LIF is also produced by Ehrlich ascites cells, buffalo rat liver cells, T-lymphocytes, or spleen cells (3), and has pleiotropic actions on cells other than hemopoietic cells. Another differentiation-inducing factor for M1 cells is interleukin 6 (IL-6) (4). IL-6 is also a pleiotropic cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, keratinocytes, macrophages, T-lymphocytes, mast cells, and many tumor cell lines (5).

Although the amino acid sequences of LIF and IL-6 show no similarity (6, 7), several functions of these cytokines, including the differentiation-inducing activity for M1 cells are similar. Both cytokines are produced by macrophages stimulated by endotoxins (8, 9), and they enhance IL-3-dependent proliferation of multipotential hemopoietic progenitors (10, 11). In embryogenesis, LIF inhibits pluripotential embryonic stem cell differentiation (12, 13), and IL-6 also has similar inhibitory activity on the differentiation of F9 mouse teratocarcinoma cells (14). LIF and IL-6 both induce differentiation in neuronal cells (15, 16) and stimulate the production of acute phase proteins in hepatocytes (17).

These overlapping biological activities suggest that the pathways of signal transduction for LIF and IL-6 may be interrelated, either at their receptors or through intracellular signaling. IL-6R is expressed in a variety of cell types in accordance with the multifunctional properties of IL-6 (5, 18, 19). Human (20) and murine IL-6R (21) were cloned, and they belong to the cytokine receptor family. A membrane glycoprotein, gp130, was first discovered as an associate protein for IL-6R (22). gp130 does not bind to IL-6, but makes a high affinity receptor when associated with low affinity IL-6R in the presence of IL-6 (23). On the other hand, cDNAs of human and murine IL-6R were cloned, and their transmembrane and cytoplasmic regions were shown to have high homologies to those of gp130 (24). Very recently, gp130 was shown to convert a low affinity LIF-R to a high affinity receptor, which indicates that gp130 is shared between IL-6R and LIF-R (25). So, it is highly plausible that the above-mentioned overlapping biological activities of LIF and IL-6 may be due to the sharing of the signal transducer, gp130.

The M1 cell line is a good model to clarify how LIF and IL-6 induce similar cell response and how LIF-R and IL-6R participate in the signal transduction. In this study, we examined the effect of LIF on the expression of IL-6R in M1 cells during differentiation. We showed that the number of high affinity IL-6R was decreased on the cell surface by treatment of the cells with LIF mainly through the down-regulation of the IL-6R mRNA level, which was also observed when the cells were treated with IL-6. We also discussed other mechanisms for the loss of high affinity IL-6R on the cell surface, including the interaction of LIF-R, IL-6R, and gp130.
MATERIALS AND METHODS

Cell Lines and Culture Conditions—M1 cells are leukemia myeloblasts that were established from spontaneous myeloid leukemia of SL strain mice in our laboratory in 1989 (26). M1 cells, clone T22-3, used in this study, were donated by Dr. M. Hozumi (Saitama Cancer Center Research Institute, Saitama, Japan). The cells were maintained at 37 °C under 5% CO2, 95% air in Dulbecco’s modified Eagle’s medium (GIBCO Laboratories) supplemented with 10% heat-inactivated horse serum (Cell Culture Laboratories, Cleveland, OH). Clone T22-3 cells are highly sensitive to LIF and IL-6 (27, 28), and differentiate into macrophage-like cells.

Cytokines and Other Reagents—Recombinant murine leukemia inhibitory factor (1 × 108 units/mg) was purchased from AMRAD Co., Ltd. (Melbourne, Australia). The specific activity of recombinant human IL-6 (hIL-6, 5 × 109 units/mg, Tosoh Corp., Kanagawa, Japan) was assessed in terms of cell stimulatory activities using a B cell line, SKW6-CL4 (29). Recombinant murine IL-6 (mIL-6) for iodination (carrier-free) was a gift from Dr. G. M. Fuller (University of Alabama at Birmingham, Birmingham, AL) and the Molecular Genetics and Protein Chemistry Group at Pfizer Inc. (Groton, CT) (30). Recombinant mIL-6 with carrier bovine serum albumin was purchased from Genzyme Corp. (Cambridge, MA). Recombinant murine tumor necrosis factor-α (TNF-α) was provided by the Suntory Institute for Biomedical Research (Osaka, Japan). Active forms of vitamin D hormone (1α,25(OH)2D3) and dexamethasone were purchased from Duophar Co. (Weesp, Netherlands) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively.

Iodination of IL-6 and TNF-α—125I-hIL-6 was prepared as described by Bolton and Hunter (31) with some modifications. 10.4 × 106 units/mg of IL-6 (20 pg/lane) was dissolved in 50 μl of 0.1 M borate buffer, pH 8.5, was added to 1.34 nCi of carrier-free hIL-6 (Du Pont-New England Nuclear), and the reaction mixture was incubated on ice for 2.5 h. The reaction was stopped by adding 500 μl of 0.2 M glycine in borate buffer, followed by further incubation on ice for 15 min. 125I-hIL-6 was separated from 125I by gel filtration, using a Sephadex G-15 column (PD-10, Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, containing 0.25% gelatin, and stored at 4 °C.

Recombinant mIL-6 was labeled with Na125I using IODO-GEN (Fierce Chemical Co.) as described by Nesbitt and Fuller (32) with some modifications. 250 μCi of Na125I in 50 μl of 0.1 M sodium phosphate buffer, pH 7.4, was added to a glass tube coated with 20 μg of IODO-GEN and incubated for 8 min on ice. The solution was then transferred to a tube containing 5 μg of recombinant mIL-6 and allowed to react for 5 min on ice. The solution was applied onto a PD-10 column equilibrated with 0.05 M sodium phosphate buffer, pH 7.4, containing 0.25% gelatin, and the material eluted at void volume was stored at 4 °C.

Iodinated hIL-6 and mIL-6 had a specific activity of 0.53–2.5 × 106 cpm/nCi, which was determined by self-displacement analysis (33) and retained the same differentiation-inducing activity for M1 cells, as compared to that of unlabeled hIL-6 and mIL-6 when subjected to SDS-15% polyacrylamide gel electrophoresis and confirmed to be a single band with a molecular mass of 21,000 daltons. Recombinant murine TNF-α was labeled with Na125I using IODO-GEN. 500 μCi of Na252I in 50 μl of 0.1 M sodium phosphate buffer, pH 7.4, was added to a glass tube coated with 20 μg of IODO-GEN and incubated for 8 min on ice. The solution was then transferred to a tube containing 5 μg of recombinant murine TNF-α and allowed to react for 5 min on ice. 125I-TNF-α was separated from 125I-labeled low molecular weight products using a gel filtration column (PD-10, Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.05 M sodium phosphate buffer, pH 7.4, containing 0.25% gelatin, and the material eluted at void volume was stored at 4 °C.

Iodinated TNF-α had a specific activity of 0.53–2.5 × 106 cpm/nCi, which was determined by self-displacement analysis (33) and retained the same cytolytic activity as unlabeled TNF-α when assayed to the culture dish after 48 h treatment with LIF or IL-6.

RESULTS

Time Course of 125I-IL-6 Binding on M1 Cells Induced by LIF or IL-6—The M1 cell differentiation-inducing activity of hIL-6 was examined after 48 h of incubation with various concentrations of cytokines by determining the phagocytosis activity of latex particles as a differentiation marker (37). Phagocytic cells increased dose dependently and reached the maximum levels (70–80%) after the administration of 150 units/ml of LIF or 400 units/ml of IL-6 (data not shown).

We examined the expression of IL-6R in M1 cells by using 125I-labeled hIL-6 when cells were induced to differentiate by treatment with LIF or IL-6 (Fig. 1A). Cells were cultured with these cytokines in suspension. Cells became to be adherent to the culture dish after 48 h treatment with LIF or IL-6, but cells were not attached to the dish after incubation with or without cytokines. Cell growth was similar between untreated and IL-6 or IL-6-treated M1 cells (doubling time, ~24 h).

Cells were incubated with 150 units/ml of LIF to induce maximal differentiation, and specific binding of 125I-hIL-6 to M1 cells was determined at various time points. Closed circles in Fig. 1A show the rapid decline in labeled hIL-6 binding. The decrease in binding was observed at 1 h after LIF treatment (about 20%) (data not shown).

When the cells were incubated with 400 units/ml of hIL-6 measured in a y-counter (1250 Multigamma II, LKR-WALLAC Instruments, Turku, Finland). All binding assays were performed in duplicate.

Determinations of the Half-life of Surface IL-6R—Cells were preincubated with 30 μg/ml of cycloheximide (CHX) at 37 °C for 30 min. Then the cells were incubated with or without 150 units/ml of LIF for various periods in the presence of CHX, and the specific binding of 125I-hIL-6 was determined as described above.

Northern Blot Analysis—Total cellular RNA was prepared from untreated M1 cells or M1 cells treated with LIF or IL-6 for various times. RNA was also prepared from M1 cells treated with IL-6 alone and dexamethasone. Poly(A)+ RNA was isolated from total RNA of untreated M1 cells using oligo(dT)-cellulose beads (Oligo-xt-DT30, Daiichi Pure Chemicals, Tokyo, Japan). Total RNA (20 μg/lane) or poly(A)+ RNA (4 μg/lane) was separated on 1% agarose-formaldehyde gel, transferred to a nylon membrane (GeneScreen Plus, Du Pont-New England Nuclear) and hybridized with a 32P-labeled mouse IL-6 cDNA probe in 5 × standard saline citrate (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 50% deionized formamide, 3 × Denhardt’s solution (3 × Denhardt’s: 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.002% Ficoll), 1% SDS, and 400 μg/ml of sonicated-denatured salmon sperm DNA at 42 °C for 16 h. The filters were washed with 2 × SSC at room temperature and 2 × SSC containing 0.5% SDS at 65 °C. pMR301 containing mouse IL-6 cDNA was a kind gift of Dr. T. Hirano (Biomedical Research Center, Osaka University Medical School, Osaka, Japan). M1-6 was used to prepare the standard curve.

RESULTS

Time Course of 125I-IL-6 Binding on M1 Cells Induced by LIF or IL-6—The M1 cell differentiation-inducing activity of hIL-6 was examined after 48 h of incubation with various concentrations of cytokines by determining the phagocytosis activity of latex particles as a differentiation marker (37). Phagocytic cells increased dose dependently and reached the maximum levels (70–80%) after the administration of 150 units/ml of LIF or 400 units/ml of IL-6 (data not shown).

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Cells were incubated with 150 units/ml of LIF to induce maximal differentiation, and specific binding of 125I-hIL-6 to M1 cells was determined at various time points. Closed circles in Fig. 1A show the rapid decline in labeled hIL-6 binding. The decrease in binding was observed at 1 h after LIF treatment (about 20%), and the maximal effect was observed at 3–6 h after treatment (70–80%). Binding then slowly decayed up to 24 h but decreased slightly again at 48 h.
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FIG. 1. Time course of specific binding of 125I-IL-6 to M1 cells treated with several inducers. A, M1 cells (4 × 10^6/ml) were incubated with 150 units/ml of LIF (closed circles), 400 units/ml of hIL-6 (open circles), 0.5 μM of 1α,25(OH)2D3 and 1 μM of dexamethasone (closed triangles) or medium alone (closed squares) for the indicated periods and collected. 5 × 10^6 cells were incubated with 125I-hIL-6 (1 × 10^6 cpm) with or without a 200-fold excess of unlabeled hIL-6 for 2 h at 4 °C, and specific binding was determined as described under “Materials and Methods.” When the cells were incubated with hIL-6 including the zero time control cells, cold IL-6 was eluted from the surface receptors by acid treatment (150 mM NaCl, 50 mM glycine HCl, pH 3.0, at 4 °C for 1 min) before the binding assay. The results were expressed as a percentage of specific binding of untreated cells. The data were the average and the standard deviation of three independent experiments with similar results. B, time course of 125I-TNF-α binding to M1 cells. M1 cells were incubated with 150 units/ml of LIF for the indicated periods and collected. 5 × 10^6 cells were incubated with 125I-TNF-α (1 × 10^6 cpm) with or without a 200-fold excess of unlabeled TNF-α for 2 h at 4 °C, and specific binding was determined as described under “Materials and Methods.” The data were the average and the standard deviation of three independent experiments with similar results.

(open circles), which induced maximal differentiation, the decrease in 125I-hIL-6 binding was more rapid. The binding of labeled IL-6 became almost undetectable at 3 h (99% decrease), and then increased and decreased as was observed after incubation with LIF. This decrease in binding was not merely caused by the occupation of receptors by unlabeled IL-6 added to the culture, because unlabeled IL-6 bound to the receptors was removed by acid treatment prior to the binding assay. We examined and confirmed that acid treatment did not affect the specific binding of 125I-hIL-6, whereas this treatment slightly increased the nonspecific binding by ~1.3 fold. This result indicates that the acid treatment did not damage or alter the cell surface receptors. This decrease is attributable to the internalization of IL-6R following the binding of exogenously added IL-6 as was observed in the other cytokine receptors (38, 39).

Although M1 cell differentiation is reported to be induced by treatment with the active form of vitamin D3 (1α,25-(OH)2D3) or dexamethasone (40, 41), the response of the M1 T22-3 clone, which we used, to these factors was weak even in the presence of excess amounts. However, M1 cells fully differentiated into macrophage-like cells after 48 h (66%, data not shown) when these factors were added together (0.5 μM of 1α,25(OH)2D3 and 1 μM of dexamethasone). Other cytokines such as granulocyte colony-stimulating factor (G-CSF) and IL-1 are known to be induced differentiation in M1 cells (42, 43), but our M1 T22-3 clone hardly responded to these factors. When the cells were incubated with 0.5 μM of 1α,25(OH)2D3 and 1 μM of dexamethasone (closed triangles; culture condition and cell viability were the same as with LIF- or IL-6 treatment), a transient increase in 125I-hIL-6 binding was observed at 6 h, but the binding returned to the basal level at 12 h and showed no obvious change for up to 48 h. Such an increase in the 125I-IL-6 binding was also observed in hepatocytes stimulated with dexamethasone (32, 44). This indicates that an LIF-induced decrease in 125I-hIL-6 binding to M1 cells is not a consequence of cell differentiation. 125I-hIL-6 binding did not change by incubation with medium alone over 48 h (closed squares). The down-regulation of IL-6R by the treatment with LIF and IL-6 was found to be statistically significant (p < 0.0001 by two-way ANOVA).

To examine whether LIF induces the decrease in other cytokine binding, the binding of 125I-TNF-α to M1 cells was also examined (Fig. 1B). The receptors of TNF-α are reported to be expressed in M1 cells (34). Fig. 1B shows the time course of 125I-TNF-α binding after the treatment of M1 cells with LIF. The binding of TNF-α increased after 48 h, as reported previously (34). A decrease in the binding at earlier time points was, however, not observed.

Dose dependence of the LIF-induced decrease in 125I-hIL-6 binding was examined. Cells were incubated with various concentrations of LIF for 4 h, the period found to produce the maximal decrease in the previous tests (Fig. 1A, closed circles). The maximal decrease in 125I-labeled hIL-6 binding was observed when the cells were incubated with 150 units/ml of LIF, resulting in the loss of about 80% of binding (Fig. 2, closed circles). From these results, we fixed the concentration of LIF to 150 units/ml in the following experiments. 125I-labeled TNF-α binding was not changed by LIF treatment (Fig. 2, open circles).

Decreased Binding of 125I-IL-6 Resulted from a Decrease in the Number of High Affinity Receptors—After 24 h of exposure

FIG. 2. Dose dependence of the LIF-induced decrease in 125I-IL-6 binding. M1 cells were incubated with various concentrations of LIF for 4 h and collected. The specific binding of 125I-hIL-6 (closed circles) and 125I-TNF-α (open circles) was examined as described under “Materials and Methods.” The results were expressed as a percentage of specific binding of untreated cells.
to LIF, M1 cells acquired a considerable level of phagocytic activity (about 40%, data not shown). M1 cells treated with LIF reduced clonogenicity, and this response became evident within 6 h of exposure (45). In terms of the transduction of the differentiation signal, early events occurring within several hours were assumed to be important. Thus, we focused on the decrease in IL-6 binding observed within 6 h. To examine whether the decrease in the binding of labeled IL-6 was due to the change in the number of receptors or in their affinity, a Scatchard plot analysis was performed using 125I-hIL-6 (Fig. 3A). Untreated M1 cells had single affinity receptors ($K_d = 2.25$ nM, 509 sites/cell). Following the incubation with LIF for 1 or 6 h, the number of binding sites decreased markedly (439 and 111 sites/cell, respectively) without any change in affinity ($K_d = 2.40$ and 3.05 nM, respectively). Thus, the decrease in 125I-IL-6 binding by treatment with LIF was due to a decrease in the number of binding sites on the cell surface rather than to a change in affinity.

In the above experiments, we used 125I-labeled human IL-6 in the binding assay for murine M1 cells. Since it is necessary to use the ligand of the same species to determine the true affinity of IL-6R on M1 cells, a Scatchard plot analysis using 125I-labeled murine IL-6 was performed next (Fig. 3B). Untreated M1 cells again had a single class of IL-6R, and the affinity was 10-fold higher than that detected by 125I-hIL-6 ($K_d = 290$ pm), which can be referred to as high affinity receptors. The affinity did not change after 1 and 6 h of LIF treatment ($K_d = 211$ and 325 pm, respectively). The number of high affinity receptors decreased from 720 sites/cell in untreated M1 cells to 644 and 292 sites/cell in M1 cells treated with LIF for 1 and 6 h, respectively. This was consistent with the results shown in Fig. 3A, and the time course of 125I-mIL-6 binding after LIF treatment for over 48 h was also similar to that detected by 125I-hIL-6 in Fig. 1A (data not shown).

### Loss of Cell Surface High Affinity Receptors Was Not Caused by the Binding of Endogenously Produced IL-6

—Undifferentiated M1 cells were reported to produce low levels of IL-6 constitutively. This production is also reported to be enhanced during differentiation, but the amount of IL-6 secreted into culture media was too low to induce M1 cell differentiation (46). It may be possible, however, that the low levels of IL-6 cause receptor down-regulation. We examined the effect of endogenously produced IL-6 on the down-regulation of IL-6R. Fig. 4 shows the mIL-6 activity in the culture medium of M1 cells incubated with or without LIF. Hybridoma 7TD1 cells were used for the titration of IL-6 using recombinant murine IL-6 as a standard, as described under "Materials and Methods." In our assay, as little as 0.4 pg/ml of mIL-6 could be detected. We confirmed that 7TD1 cells did not proliferate in response to as much as 150 units/ml of LIF (data not shown). When M1 cells were incubated in the medium alone (Fig. 4, open circles), IL-6 activity was first detected after 6 h (2.0 pg/ml), but the level remained lower than 10 pg/ml even after prolonged incubation for 48 h (8.0 pg/ml). When the cells were incubated with 150 units/ml of LIF (Fig. 4, closed circles), IL-6 activity also became detectable after 6 h (5.8 pg/ml), increasing in the supernatant to 168 pg/ml at 48 h. It should be noted that IL-6 activity was undetectable for the initial 3 h.

The minimum concentration of murine IL-6 required to cause the decrease in 125I-IL-6 binding was determined by incubating cells for 4 h with various concentrations of unlabeled mIL-6 (Fig. 5). The binding of 125I-hIL-6 did not change when incubated with up to 50 pg/ml of mIL-6 and then decreased dose dependently. 125I-hIL-6 binding was determined every hour for 4 h following the addition of 25 pg/ml of mIL-6, and again, no decrease in 125I-IL-6 binding was observed (data not shown). These results suggested that the amount of endogenously produced IL-6 was insufficient to induce the loss of high affinity receptors for at least the first 6 h after LIF treatment.

We further examined the effect of anti-murine IL-6 antibody (Genzyme Corp.) on the LIF-induced decrease in 125I-IL-6 binding. The neutralizing activity of the antibody was...
confirmed by the 7TD1 titration system. Enough excess antibody to neutralize IL-6 secreted into culture media inhibited the standard deviation shown), which supports the previous conclusion that the loss of high affinity IL-6R is not caused by endogenously produced IL-6.

Expression of IL-6R Was Regulated at the mRNA Level—We examined whether the loss of high affinity receptors was attributable to the regulation of receptor expression. The IL-6R mRNA level was examined in M1 cells treated with LIF or hIL-6 for various periods by Northern blot analysis (Fig. 6A, lanes 2–16). Using a full length cDNA encoding mIL-6R, we detected transcripts of 5–6 kb. Fig. 6B shows the densitometrical analysis of the transcripts. Regardless of whether the cells were treated with LIF or IL-6, a similar down-regulation of IL-6R mRNA was observed. After 3 h of incubation with either LIF or IL-6, the level of IL-6R mRNA reached its nadir, and then began to increase. At 24 h, mRNA recovered to its preadministration level and continued to increase until 48 h. This increase in the mRNA levels of IL-6R was more prominent in IL-6-treated cells than in LIF-treated cells.

The time course of the level of cell surface receptors examined by 

When the differentiation of M1 cells was induced by the treatment with 1α,25(OH)2D3 and dexamethasone, a transient increase in IL-6 mRNA was observed (Fig. 6A, lanes 17 and 24), but returned to the initial level at 12 h (Fig. 6A, lanes 23–25). Such an increase in the IL-6R mRNA level was also observed in hepatocytes stimulated with dexamethasone (32, 44), and this regulation is in accordance with the changes in the 125I-IL-6 binding on the cell surface (see Fig. 1A, closed triangles). Thus, a decrease in the IL-6R mRNA level when treated with LIF or hIL-6 is not merely a consequence of cell differentiation. When cells were incubated with medium alone, the IL-6R mRNA level did not change (Fig. 6A, lanes 17–21).

Regulation of mRNA Was Not the Only Mechanism Responsible for the Loss of High Affinity Receptors on the Cell Surface—The half-life of IL-6R was analyzed next, as described under “Materials and Methods,” to examine the effect of treating the cells with LIF. We investigated the possibility that other mechanisms were responsible for the loss of the high affinity receptors. When M1 cells were preincubated with 30 μg/ml of CHX to block new protein synthesis, 125I-hIL-6 binding decreased on the cell surface with a half-life of 30 min (Fig. 7, open circles). LIF was introduced after 30 min of preincubation with CHX (at time 0), when protein synthesis was considered to be completely blocked. The decrease in the binding on the cell surface was more rapid after LIF treatment with a half-life of 16 min (Fig. 7, closed circles). This suggests that LIF decreases surface high affinity IL-6R not only by suppressing their production, but also by decreasing the half-life.
we showed that treatment of M1 cells with LIF at 37 °C decreased the number of cell surface high affinity IL-6R. Affinity of $^{125}$I-labeled human IL-6 was 10 times lower for IL-6R on M1 cells than that of $^{125}$I-labeled murine IL-6. The number of IL-6R was almost the same either by the detection with $^{125}$I-hIL-6 or with $^{125}$I-mIL-6, and the decrease in the number of IL-6R was similarly observed by either labeled ligand (Fig. 3). This decrease is not merely a consequence of cell differentiation nor a generalized phenomenon by LIF treatment, because the decrease in $^{125}$I-IL-6 binding was not caused by the treatment with $\alpha_25(OH)_2D_3$ and dexamethasone, and because $^{125}$I-TNF-α binding was not changed after LIF treatment, either.

The time course of IL-6R expression on the cell surface and that of IL-6R mRNA level are quite similar until 12 h after LIF treatment; a decrease becomes apparent within 3 h, and the levels recover after 6-12 h. The level of IL-6R mRNA reaches its nadir at 3 h after treatment, whereas the amount of ligand binding on the cell surface is lowest at 6 h. This difference may be attributed to the time lag required for the translation and transport of IL-6R to the cell surface. From these results, the decrease in the number of high affinity IL-6R observed at early stages of LIF treatment is most probably controlled at the mRNA level.

At 24-48 h, receptor expression on the cell surface did not recover to expected levels based on the mRNA level (compare Fig. 1A and Fig. 6B). This is not an artifact by dead cells, because cell viability was more than 95% even after 48 h of incubation with LIF. This may be due to the internalization of receptors caused by the increased amounts of IL-6, which is endogenously produced and secreted by differentiated cells (Fig. 4), but the addition of excess anti-mIL-6 antibody into the culture media did not affect the decrease in $^{125}$I-IL-6 binding (data not shown). It is possible that IL-6 and IL-6R bind intracellullarly and that the binding sites of receptors are already occupied before the receptors are exposed on the cell surface. However, this possibility seems not to be the case, because the level of $^{125}$I-IL-6 binding on M1 cells incubated with LIF for 48 h remained unchanged even when the cells were washed with acid buffer prior to the binding assay in order to elute ligands from the surface receptors (data not shown). When the cells were induced to differentiate with $\alpha_25(OH)_2D_3$ and dexamethasone, both $^{125}$I-IL-6 binding on the cell surface and the IL-6R mRNA level at 24-48 h was the same as untreated cells. The cause of such a difference of IL-6R expression by different inducers is not clear, but the phenotype of 48-h-treated cells might be different, although the phagocytosis activity as a differentiation marker is at the same level. As M1 cells show drastic changes in cell size, shape, and functions after differentiation, it is conceivable that the equilibrium between receptor production and its disappearance from the cell surface, including shedding of receptors from the cell surface as is observed in other cytokine receptors, is different between untreated M1 cells and fully differentiated cells, which remains to be examined in future.

A number of cytokine receptors have been cloned, and the regulation of their expression has been investigated. Posttranscriptional regulation of M-CSF-R mRNA by GM-CSF or multi-CSF (IL-3) is reported (55), in accordance with the lineage restriction activity of GM-CSF or multi-CSF. The up-regulation of IL-6R mRNA by glucocorticoid has been observed on human hepatoma cell lines in accordance with the stimulation of the synthesis of acute phase proteins by the synergistic action of glucocorticoid and IL-6 (32, 44). GM-CSF down-regulates IL-6R mRNA in human polymorphonuclear leukocytes, although the effect of IL-6 on these cells is not well understood (56).

It was reported that in normal human blood monocytes, IL-6 down-regulated IL-6R mRNA during cell maturation into macrophages (57). In our study, both LIF and IL-6 down-regulated IL-6R mRNA in M1 cells. IL-6R rapidly disappeared on the cell surface after treatment with IL-6 (Fig. 1A), possibly because of the internalization of the receptors. In addition to this internalization, a decrease in the rate of receptor supply to the cell surface may contribute to the down-
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regulation. It is likely that both LIF and IL-6 induce the similar down-regulation of IL-6R mRNA through the common signal transducer gp130, which is shared between LIF-R and IL-6R. The biological meaning of this down-regulation of IL-6R mRNA may be considered as follows. Only a limited number of receptors on the cell surface is required to transmit the differentiation signal into the cells, and once enough ligands bind to the receptors, other receptors on the cell surface are no longer necessary. Moreover, the rapid decrease in the number of receptors on the cell surface may prevent unnecessary overbinding of the ligands, resulting in the preservation and efficient utilization of the ligands. Whether the down-regulation of cytokine receptor mRNA during differentiation occurs in other cytokine receptors or other cell lines should be examined to clarify the biological significance.

The half-life of surface IL-6R was 30 min when examined in the presence of CHX. The addition of LIF shortened the half-life to 16 min, which indicates that the decrease in IL-6 binding observed in Fig. 1A and the decrease in the number of high affinity IL-6R observed in Fig. 3 may not be solely due to a decrease in the receptor biosynthesis. After 1 h of LIF treatment, the IL-6R mRNA level did not decrease (Fig. 6), but IL-6R on the cell surface detected by the binding assay exhibited about a 20% decline (Fig. 1A). This apparent discrepancy might be explained by the reduction of the half-life of cell surface IL-6R by LIF treatment. One possible mechanism that explains this reduced half-life may be the acceleration of the shedding of receptors from the cell surface into the culture media. Shedding of cytokine receptors has been reported for IL-2 (53) and TNF (54) receptors. Soluble IL-6R is also detected in normal human urine (58). However, our preliminary data showed that the amount of soluble IL-6R in the culture medium of M1 cells was undetectable at least for 6 h of LIF treatment using enzyme-linked immunosorbent assay analysis (data not shown, see Ref. 59), which is against the shedding of IL-6R from the cell surface.

Another possible explanation for the reduced half-life of IL-6R by LIF treatment is receptor-receptor interaction on the cell surface. Hibi et al. (23) reported that K
\(_d\) values of high and low affinity IL-6R were 40 pm and 5.3 nm in T cell line transfected with IL-6R cDNA. Gearing et al. (25) reported that K
\(_d\) values of high affinity IL-6R were 27 and 35 nm in COS-7 cells transfected with IL-6R cDNA and gp130 cDNA and that only high affinity receptor (K
\(_d\) = 310 pm) was detected in B9 cells. In our study, the affinity of IL-6R of M1 cells was 200 pm when examined using 125I-labeled murine IL-6 (Fig. 3B), and this K
\(_d\) value suggests that IL-6R on the M1 cell surface may associate with gp130, resulting in the formation of high affinity receptors. Thus, the decrease in half-life of IL-6R by LIF treatment is receptor-receptor interaction on the cell surface. Hibi et al. (23) reported that K
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