Interleukin-1-induced Growth Inhibition of Human Melanoma Cells

INTERLEUKIN-1-INDUCED ANTIZYME EXPRESSION IS RESPONSIBLE FOR ORNITHINE DECARBOXYLASE ACTIVITY DOWN-REGULATION*

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Interleukin (IL)-1 is a multi-functional cytokine and regulates cell growth either positively or negatively. Previous studies have shown that IL-1-induced ornithine decarboxylase (ODC) activity down-regulation is involved in the anti-proliferative effect of IL-1 on human A375 melanoma cells. In this study, we examined the IL-1α-induced molecular events resulting in ODC activity down-regulation in C2-1, a A375 cell line stably transfected with human type I IL-1 receptor. Recombinant human (rh) IL-1α inhibited the growth and down-regulated the ODC activity of C2-1 cells in a dose-dependent manner. Kinetics studies showed that both the DNA synthesis and ODC activity of C2-1 cells progressively decreased from 12 h after IL-1 addition. Northern hybridization showed that IL-1 had no influence on ODC mRNA level. However, rhIL-1 induced both a decrease of ODC protein and an ODC-inhibiting activity in IL-1-treated C2-1 cells. IL-1 specifically up-modulated the mRNA level of antizyme, a protein essential for ODC regulation, but had little effect on its stability. IL-1-induced antizyme up-modulation preceded IL-1-induced down-regulation of ODC protein, ODC activity, and DNA synthesis in C2-1 cells. Run-on transcription analysis confirmed that the increased antizyme mRNA expression was due to elevated antizyme gene transcription. Furthermore, the action of IL-1 to inhibit the ODC activity and growth of C2-1 cells was blocked by expressing the antisense RNA of human antizyme in C2-1 cells. These results suggest that IL-1-induced antizyme expression is responsible for IL-1-induced ODC activity down-regulation in human melanoma cells.

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‡ The abbreviations used are: IL, interleukin; AZ, antizyme; hAZ, human AZ; ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; GAPDH, glyceraldehyde phosphate dehydrogenase; IL-1R, interleukin-1 receptor; rh, recombinant human; TPA, 12-O-tetradecanoylphorbol 13-acetate; NC, nitrocellulose; ELISA, enzyme-linked immunosorbent assay; bp, base pair(s).

Interleukin-1 (IL-1) is one of the multi-functional cytokines produced predominantly by activated monocytes and macrophages and participates in many host reactions (1, 2). It has been not only incriminated in various disease states such as endotoxic shock (3) and autoimmune diseases (4) but also shown to play beneficial roles in immune defense, homeostasis, and immune surveillance (1, 2, 5). In vitro, IL-1 regulates cell growth in either a positive or a negative manner. With respect to its negative effect on cell growth, IL-1 has been reported to suppress the growth of a variety of normal and malignant cell types including pancreatic Langerhans cells (6), endothelial cells (7), and tumor cell lines derived from melanoma (8), breast carcinoma (9), myeloid leukemia (10), ovarian carcinoma (11), and lung adenocarcinoma (12).

Two agonist species of the IL-1 family, IL-1α and IL-1β, although encoded by different genes and sharing only 26% amino acid sequence homology (13), can bind the same cell surface receptors and mediate similar biological actions (14). Two types of IL-1 receptors (IL-1R) have until now been identified: type I IL-1R is an 80-kDa glycoprotein preferentially expressed on fibroblasts and T cells (15, 16), whereas type II IL-1R is a 60-kDa glycoprotein primarily present on B cells, macrophages, and neutrophils (17). Only type I IL-1R can transduce IL-1 signal, whereas type II IL-1R works as a decoy receptor to dampen signaling via type I IL-1R (18, 19). The binding of IL-1 to type I IL-1R has been proposed to cause activation of different second messenger pathways including activation of a GTP-binding protein with no associated increase in adenyl cyclase (20), activation of adenyl cyclase (21, 22), hydrolysis of three phospholipids by nonphosphatidylinositol phospholipase Cs (23, 24), release of ceramide from sphingomyelin after activation of sphingomyelinase (25), and release of arachidonic acid from phospholipids via cytosolic phospholipase A2 after its activation by phospholipase A2-activating protein (26, 27). Very recent studies suggest the involvement of either activation of mitogen-activated protein kinases and tyrosine kinases or down-regulation of protein phosphatase (28–32). However, the precise transmembrane and/or intracellular pathway(s) for different aspects of IL-1 action has not been fully elucidated.

The first intracellular alteration probably associated with the anti-proliferative effect of IL-1 in A375 human melanoma cells was reported to be down-regulation of ornithine decarboxylase (ODC) activity (34). Subsequently, IL-1-induced IL-6 production was shown to mediate in part the anti-proliferative effect of IL-1 on A375 melanoma cells (35). IL-1 treatment also caused A375–6 cells to be arrested in G0/G1 phase of the cell cycle (36). Furthermore, studies documented by Rangnekar et al. emphasized the importance of an IL-1-induced early gene expression program (38), and they proposed that IL-1-induced gro (37) and Egr-1 (39) genes expression might be associated with IL-1-induced growth arrest in A375 cells.

Very recently, we transfected A375–5 cells, a twin subclone of A375–6 expressing no detectable IL-1R (33), with a human
type I IL-1R cDNA and obtained a series of IL-1R-positive stable transfectants (40). Among these transfectants, some were IL-1-sensitive and others were IL-1-resistant. The most obvious difference between the two categories of transfectants was that upon IL-1 treatment, all the sensitive transfectants down-regulated their ODC activities, whereas all the resistant transfectants failed to do so. These results combined with the observation that putrescine, a resultant product of ODC reaction, reversed the anti-proliferative effect of IL-1 (40), and our previous studies (34, 36) led us to conclude that IL-1-induced ODC activity down-regulation plays a very essential role in the anti-proliferative effect of this cytokine on A375 melanoma cells (40). However, how ODC activity is down-regulated by IL-1 remains largely unknown. In the present study, we addressed this question by the use of C2-1, an IL-1-sensitive transfectant A375 cell line (40). Our results suggest that IL-1-induced up-modulation of AZ expression is responsible for IL-1-induced ODC activity down-regulation in C2-1 melanoma cells.

MATERIALS AND METHODS

Reagents—RPMI 1640 and TPA were purchased from Sigma. Fetal bovine serum was purchased from Beckne (Toronto, Canada). Recombinant human IL-1α (rhIL-1α, 2 × 10^5 units/mg) and rh tumor necrosis factor α (10^5 units/mg) were provided by Dr. T. Yamada (Dainippon, Osaka, Japan). Recombinant human IL-6 was provided by Dr. Y. Akiyama (Ajinomoto, Yokohama, Japan). Recombinant human interferon α was obtained from Nippon Roche (Kamakura, Japan). Oncosta- tin M was a generous gift from Dr. C. Reynolds (NCI, National Institutes of Health, Frederick, MD). tRNA(14C)Ornithine hydrochloride (56 mM/C/ml) was purchased from Amersham Corp. Rabbit polyclonal anti-HOD IgG antibodies and mouse monoclonal anti-hODC IgG antibodies (41) were generous gifts from Drs. A. Kadota and K. Nakayama (Idemitsu Kosan Co., Ltd., Sodegaura, Chiba, Japan).

Cell Culture—The C2-1 cell line, a stable hIL-1RI transfectant of A375–5 human melanoma cells (40), was maintained in culture medium (RPMI 1640 supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, 15 mM HEPES, and 5% fetal bovine serum) at 37 °C in a humified atmosphere containing 5% CO2. The C2-1 cells were detached with 0.05% trypsin-0.02% EDTA in phosphate-buffered saline. After washing with culture medium and being adjusted to 2 × 10^5 cells/ml, C2-1 cells were distributed into Falcon 3003 dishes (Becton Dickinson) at a volume of 10 ml/dish. After preincubation for 2 days to allow complete recovery of cells from trypsinization, the culture supernatants were replaced by prewarmed fresh culture medium in the absence or the presence of different concentrations of rhIL-1α or other reagents at different concentrations as specified.

Assays for Growth and DNA Synthesis—Cells in suspension (4 × 10^4 cells/ml) were distributed into 96-well flat-bottomed plates (Falcon, Lincoln, NJ) at 0.1 ml/well and then subjected to incubation for 24 h at 37 °C under humidified atmosphere containing 5% CO2. Therefore, 0.1-ml aliquots of culture medium in the absence or the presence of different concentrations of rhIL-1α were added in triplicate, and the cells were cultured for different periods of time as specified. Cell proliferation was determined by crystal violet stain as described previously (40). The cell pellet was resuspended in 0.1 ml of storage buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, and 40% glycerol, v/v). The amplified fragment was cloned into the EcoRI site of pGEM-3Z to create pGEM-hODC, and the fidelity of the cloned fragment was confirmed by sequencing; (ii) a 550-bp cDNA fragment of human AZ that was also amplified by reverse transcribe-polymerase chain reaction method using A375–5 cell-derived mRNA as template. The amplified fragment was cloned into pT7Bluehyb vector and transfected into Escherichia coli (3-3) and 5′-CTCGGAGATTTCTGGGACCACGGC-3′ and 5′-CTGGCAGATTTCGTGGGACCCG-3′. The fragment obtained was cloned into the EcoRI site of PGEM-3Z to create pGEM-hODC. After hybridization, the filters were washed twice at room temperature in 2 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) for 30 min. The filters were then soaked in 0.5N NaOH and measured with a fluid scintillation counter (LSC-1000; Aloka, Tokyo, Japan).

Mechanism(s) of IL-1-induced ODC Activity Down-regulation—IL-1-induced ODC activity down-regulation in C2-1 melanoma cells was established by the use of C2-1, an IL-1-sensitive transfectant A375 cell line (40). Our results suggest that IL-1-induced up-modulation of AZ expression is responsible for IL-1-induced ODC activity down-regulation in C2-1 melanoma cells.

Total DNA Extraction and Northern Hybridization—Total DNA was extracted from monolayer cultures of C2-1 cells according to Chomczynski and Sacchi (43). After size fractionation on a agarose-formaldehyde gel and transfer to a nitrocellulose (NC) filter, the specific mRNA on the filter was detected by hybridization with a 32P-labeled cDNA probe at 42 °C for about 17 h in hybridization buffer comprising 50% formamide, 5 × SSPE (1 × SSPE is 0.15 mM NaCl, 10 mM NaF, 10 mM EDTA, pH 7.4), 5 × Denhardt’s solution, 1% SDS, and 100 μg/ml denatured salmon sperm DNA. The following probes were used: (i) a 611-bp cDNA fragment corresponding to bases 2–612 of reported human ODC cDNA (44). This cDNA fragment was amplified by reverse transcriptase-polymerase chain reaction using A375–5 cell-derived mRNA as template. The amplified fragment was cloned into pT7Bluehyb vector and transfected into Escherichia coli (3-3) and 5′-CTCGGAGATTTCTGGGACCACGGC-3′ and 5′-CTGGCAGATTTCGTGGGACCCG-3′. The fragment obtained was cloned into the EcoRI site of PGEM-3Z to create pGEM-hODC, and the fidelity of the cloned fragment was confirmed by sequencing; (ii) a 550-bp cDNA fragment of human AZ that was also amplified by reverse transcribe-polymerase chain reaction method using A375–5 cell-derived mRNA as template. The amplified fragment was cloned into pT7Bluehyb vector and transfected into Escherichia coli (3-3) and 5′-CTCGGAGATTTCTGGGACCACGGC-3′ and 5′-CTGGCAGATTTCGTGGGACCCG-3′. The amplified fragment was cloned into the EcoRI site of PGEM-3Z to create pGEM-hAZ. Sequencing the cloned hAZ cDNA fragment by dideoxy terminator method showed that it corresponded to bp 186–726 of the full-length human AZ cDNA cloned from Duadi cells (49); (iii) a Pst-digested 1300-bp fragment of human glyceraldehydephosphate dehydrogenase (GAPDH) (46). The probes labeled by random priming (Multi Prime DNA Labeling Kit, Amersham Corp.). After hybridization, the filters were washed twice at room temperature in 2 × SSC (1 × SSC is 0.15 mM NaCl, 15 mM sodium citrate, pH 7.0) for 30 min followed, if necessary, by washing in 0.2 × SSC until a reasonably low background was obtained. The filters were autoradiographed using a Bio-Image analyzer (Fuji BAS 2000, Tokyo, Japan).

Nuclear Run-on Assay—Nuclei preparation and in vitro transcription was performed according to the method of Celano et al. (47) with minor modifications. Briefly, 6 × 10^6 C2-1 cells were suspended in 0.5 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, and 0.5% Nonidet P-40, v/v) and incubated on ice for 10 min. Nuclear pellets were pelleted at 1000 × g for 1.5 min in cold lysis buffer. The nuclei pellet was resuspended in 0.1 ml of storage buffer (50 mM Tris-HCl, pH 8.3, 5 mM MgCl2, 0.1 mM EDTA, and 40% glycerol, v/v), immediately snap-frozen in liquid nitrogen, and stored at −80 °C. In vitro nuclear transcription was carried out in 0.1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 80 mM KCl, 0.1 mM EDTA, and 0.5 mM dithiothreitol) containing 4 mM ATP, GTP, CTP, and 100 μl of TEB buffer (blank) or cell lysate, the mixture was incubated at 37 °C for 30 min with constant shaking. Then the reaction was stopped by the addition of 0.5 ml of 0.5 N HCl, and the mixture was shaken for another 2 h. ODC activity was determined as the release of 14C-CO2 (pmole/μg of protein), which was collected on a 4-cm2 filter paper and dried at 37 °C and measured with a fluid scintillation counter (LSC-1000; Aloka, Tokyo, Japan).
μCi of [α-32P]UTP at 30 °C for 30 min. Subsequently, the mixture was treated at 30 °C for 30 min with proteinase K (final concentration, 100 μg/ml) in the presence of 50 μg of carrier tRNA, 1% SDS, and 10 mM EDTA. RNA in the mixture was extracted by the acid guanidium phenol method (43) followed by two times of ethanol precipitation. The α-32P-labeled nuclear RNA was dissolved in hybridization buffer to a concentration of 106 cpm/ml and hybridized, under the same conditions as those of Northern hybridization, to NC filters on which a number of DNA plasmids were immobilized. The following plasmids were used: pGEM-hAZ, pUC-hMnSOD (48), pUC-hGAPDH (46), and control plasmids including pUC118 and pGEM-3Z. All plasmids were linearized by digestion with appropriate restriction enzymes and denatured by alkali treatment before blotting on to NC filter (5 μg/slot).

**Construction of the Plasmid Expressing the Antisense Transcript of hAZ and Transfection**—The 1063-bp full-length hAZ cDNA was cut out by EcoRI from pAZ7.1 reported recently (49) and cloned into the EcoRI site of the expression vector pBK-RSV by standard procedure to generate recombinant plasmid pRhAZ(AS) in which expression of the antisense transcript of hAZ was controlled by the RSV long terminal repeat. Pure pBK-RSV and pRhAZ(AS) were prepared by two cycles of CsCl untracentrifugation. For transfection, 50 μg of plasmids were electrooporated into 5 × 106 C2-1 cells in 0.5 ml of Opti-MEM I under conditions (0.4-cm cuvette, 0.31 kV, 950 microfarad) by the use of the Gene Pulser II system (Bio-Rad). Under these conditions, electroporation efficiencies were 55–65% as estimated by in situ β-galactosidase staining of pSV-β-galactosidase-transfected C2-1 cells. After electroporation, cells were immediately suspended in RPMI 1640 supplemented with 10% fetal bovine serum and incubated at 37 °C for 48 h in a humidified atmosphere containing 5% CO₂. Thereafter, cells were detached with 0.05% trypsin-0.02% EDTA in phosphate-buffered saline, washed, and used for the examination of their responses to IL-1-induced ODC activity down-regulation and growth inhibition by methods as described above.

**RESULTS**

**IL-1 Treatment Caused Growth Inhibition, Decrease in DNA Synthesis, and ODC Activity Down-regulation of C2-1 Cells**—Treatment with rhIL-1α for 3 days dose-dependently inhibited the growth of C2-1 cells (Fig. 1A) in a manner similar to those of other IL-1-sensitive A375 melanoma cells (8, 34, 40). Fig. 1B showed that the DNA synthesis of C2-1 cells was suppressed by rhIL-1α in a time-dependent manner, which occurred from 12 h after IL-1 addition. As reported previously that ODC activity down-regulation was important for IL-1-induced inhibition of growth and DNA synthesis in human melanoma cells (34, 40), we subsequently investigated the effect of IL-1 on the ODC activity of C2-1 cells. Culture of C2-1 cells at 37 °C for 48 h in the presence of various concentrations of rhIL-1α down-regu-
lated their ODC activity in a dose-dependent manner (Fig. 2A). Time course study revealed that the ODC activity of cultured C2-1 cells increased transiently within 6 h, began to decrease from between 6 and 12 h, and continued to decrease thereafter after rhIL-1α addition (Fig. 2B). At 48 h after rhIL-1α addition, the ODC activity was down-regulated to about 15% of that before rhIL-1α addition. The transient increase of ODC activity was due to medium change (refer to the legend of Fig. 2) because the similar increase was also observed in the absence of rhIL-1α (data not shown). At 12 h after rhIL-1α addition, the ODC activity of C2-1 cells was still as high as that before rhIL-1α addition (Fig. 2B), whereas the rate of DNA synthesis was reduced about 25% (Fig. 1B). This discrepancy is perhaps due to the transient increase of ODC activity, because about 25% of the ODC activity was suppressed at 12 h after rhIL-1α addition compared with highest ODC activity time point caused by medium changes.

**IL-1 Had No Effect on ODC mRNA Level in C2-1 Cells**—The effect of rhIL-1α on the ODC mRNA level of C2-1 cells was investigated by Northern hybridization (Fig. 3). Treatment with rhIL-1α ranging from 0.1 to 1000 units/ml for 48 h showed that there were no obvious alterations of ODC mRNA level in C2-1 cells (Fig. 3A), nor were there any time-dependent variations of ODC mRNA expression in C2-1 cells upon treatment with 100 units/ml rhIL-1α (Fig. 3B). In a separate experiment in which total RNA was extracted at 30 min, 1 h, and 2 h after the addition of 100 units/ml of rhIL-1α, the ODC mRNA level of cultured C2-1 cells showed no alterations (data not shown). These results suggested that IL-1 neither suppressed ODC gene transcriotion nor destabilized ODC mRNA in C2-1 melanoma cells.

**IL-1 Induced Down-regulation of ODC Protein in C2-1 Cells**—We next investigated the influence of rhIL-1α on the ODC protein level of C2-1 cells (Fig. 4). rhIL-1α down-regulated ODC protein level in C2-1 cells in a dose-dependent manner (Fig. 4A). The kinetics of rhIL-1α-induced down-regulation of ODC protein in C2-1 cells (Fig. 4B) was similar to that of rhIL-1α-induced ODC activity down-regulation as illustrated in Fig. 2B. These results suggested that IL-1-induced ODC activity down-regulation was associated with and might be a result of IL-1-induced down-regulation of ODC protein in melanoma cells.

**Existence of ODC Enzyme Inhibitor(s) in IL-1-treated C2-1 Cells**—A careful comparison of Fig. 4 with Fig. 2 revealed that IL-1-induced ODC protein down-regulation could not completely explain IL-1-induced ODC activity down-regulation, because they were not completely parallel. Therefore, we examined whether there existed IL-1-induced intracellular ODC enzyme inhibitor(s), which might be responsible for part of the IL-1-induced ODC activity down-regulation. Theoretically, if there were such inhibitor(s) in IL-1-treated C2-1 cells, mixing the lysate of IL-1-treated C2-1 cells with that of untreated C2-1 cells would suppress at least in part the ODC activity existing in the lysate of untreated C2-1 cells. In order to measure the exact ODC activity, half of the cell lysates were pretreated with DFMO at 4 μM, which was predetermined to be enough to inhibit ODC activity. The results in Table I demonstrated that the ODC activity of the IL-1-treated cell lysate was almost undetectable, and a mixture containing 50% untreated and 50% rhIL-1α-treated lysates was much lower than half of the sum of ODC activities of untreated and rhIL-1α-treated lysates, indicating that there were inhibitory activities for ODC in IL-1-treated C2-1 cells.

**IL-1 Up-regulated AZ mRNA Level in C2-1 Cells**—It is well known that AZ, a small intracellular protein that functions to inhibit ODC activity by monomerizing ODC homodimer and to down-regulate ODC protein by accelerating its degradation in 26 S proteasome, is critical in the regulation of ODC activity (49–53). We therefore investigated whether IL-1 could up-modulate the AZ expression in C2-1 cells. Treatment with various concentrations of rhIL-1α at 37 °C for 24 h showed that as little as 0.1 units/ml of rhIL-1α up-modulated AZ mRNA expression in C2-1 cells, and the action was dose-dependent (Fig. 5A). The time course study revealed that rhIL-1α-induced up-modulation of AZ mRNA in C2-1 cells occurred at around 3–6 h after rhIL-1α addition (Fig. 5B). Comparison of the kinetics of rhIL-1α-induced up-modulation of AZ mRNA (Fig. 5B) with those of rhIL-1α-induced down-regulation of ODC protein (Fig. 4B) and ODC activity (Fig. 2B) indicated that upon rhIL-1α treatment, AZ mRNA up-modulation preceded down-regulation of ODC protein and activity.

**IL-1 Activated the Transcription of AZ Gene in C2-1 Cells**—Because rhIL-1α exhibited no obvious effect on the stability of AZ mRNA in C2-1 cells (data not shown), the effect of rhIL-1α on the transcription of AZ gene was investigated by nuclear
run-on experiments. Treatment with rhIL-1α at a concentration of 100 units/ml for 12 h greatly increased (approximately 6-fold) the transcription rate of AZ gene in C2-1 cells (Fig. 6). Thereafter, cell lysates were prepared, and total protein concentration was quantitated and adjusted to 1 mg/ml. The amount of ODC enzyme protein in the cell lysate was determined with an indirect double antibody ELISA method (see details under “Materials and Methods”) and presented as absorbance at a wavelength of 490 nm. The results were the mean ± S.D. of triplicated samples.

**TABLE I**

Existence of ODC inhibitor(s) in IL-1-treated C2–1 cells

| C2–1 cell lysate | DFMO treatment | CO2 release | 4 μM | dpm/k/mg protein |
|------------------|----------------|-------------|------|-----------------|
| Untreated        |                 |             |      |                 |
| 100 μl           | 100 μl          | –           | 13,671 |
| 50 μl            | 50 μl           | –           | 618   |
| 100 μl           |                 | +           | 745   |
| 50 μl            | 50 μl           | +           | 1,068 |
| 100 μl           | 100 μl          | +           | 614   |
| 50 μl            |                 | +           | 836   |

Expression of Antisense RNA of hAZ Blocked IL-1-induced ODC Activity Down-regulation and Growth Inhibition—To further examine whether IL-1-induced up-modulation of AZ mRNA expression was responsible for IL-1-induced ODC activ-
Mechanism(s) of IL-1-induced ODC Activity Down-regulation

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...activity down-regulation, we constructed a recombinant plasmid (pRhAZ(AS)), which directed the synthesis of antisense hAZ RNA, and transfected this plasmid into C2-1 cells to see if IL-1-induced ODC activity down-regulation could be blocked (Fig. 7). Transient transfection of C2-1 cells with pRhAZ(AS) blocked about 70% of rhIL-1α-induced ODC activity down-regulation, whereas transfection with the vector plasmid pBK-RSV in an identical way had no effect (Fig. 7A). Furthermore, transfection of C2-1 cells with pRhAZ(AS) also blocked approximately 60–70% of rhIL-1α-induced growth inhibition of these cells (Fig. 7B). The failure of antisense hAZ RNA to completely block the rhIL-1α-induced ODC activity down-regulation and growth inhibition in C2-1 cells was presumably because the transfection efficiency was not 100%. These results not only provided evidence that AZ up-modulation was responsible for IL-1-induced ODC activity down-regulation but also further strengthened our view that ODC activity down-regulation was essential for IL-1-induced growth inhibition in A375 melanoma cells (40).

TPA and Other Cytokines Failed to Increase the AZ mRNA Level of C2-1 Cells—Besides IL-1, a number of cytokines including interferon (8, 55), tumor necrosis factor α (34, 35), IL-6 (35, 56), and oncostatin M (57), as well as TPA2 can suppress the growth of human melanoma cells. Because the growth of C2-1 cells could also be inhibited by these reagents (data not shown), we therefore investigated whether or not these reagents could modulate the expression of AZ mRNA in C2-1 cells. Treatment of C2-1 cells for 24 h with any of these reagents at a dose larger than their ED50 failed to induce eleva-

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...and differentiation, and cell cycle control (44, 50, 51, 57). Based on the results in Figs. 1 and 2 of the present study and previous reports, several aspects of evidence support the notion that IL-1-induced ODC activity down-regulation is an essential step in IL-1-induced growth inhibition in melanoma cells: (i) IL-1-induced ODC activity down-regulation precedes IL-1-induced inhibition of DNA synthesis and proliferation in A375 cells (34); (ii) putrescine, a physiological product of ODC reaction and precursor of polyamines, can overcome most when high doses of IL-1 are used (34, 40) and all when low doses of IL-1 are used (40) of IL-1-induced growth inhibition; (iii) in melanoma cells whose ODC activities cannot be down-regulated by IL-1, their growth also cannot be suppressed by IL-1 (40); (iv) treatment of synchronously cultured melanoma cells with IL-1 delays progression from G1/G0 to S, retards progression through G2/M of the first cell cycle, and blocks progression from G0/G1 to S of the second cell cycle (36). This consequence is compatible with IL-1-induced ODC activity down-regulation because it has been reported that ODC activity begins to be blocked after IL-1 was added (36).

DISCUSSION

Treatment with rhIL-1α inhibited the growth (Fig. 1A) and down-regulated the ODC enzyme activity (Fig. 2A) of C2-1 cells in a similar dose-dependent manner. Time course studies showed that both ODC enzyme activity and DNA synthesis of C2-1 cells were down-regulated by rhIL-1α in similar kinetics; of particular note is that they both occurred at 12 h after the addition of rhIL-1α (Figs. 1B and 2B). These results are similar to those observed in other IL-1-sensitive A375 melanoma cells (34, 40). ODC catalyzes the conversion of ornithine to putrescine, the first step and a major site of regulation of polyamine biosynthesis, and is involved in the regulation of cell growth, differentiation, and cell cycle control (44, 50, 51, 57). Based on the results in Figs. 1 and 2 of the present study and previous reports, several aspects of evidence support the notion that IL-1-induced ODC activity down-regulation is an essential step in IL-1-induced growth inhibition in melanoma cells: (i) IL-1-induced ODC activity down-regulation precedes IL-1-induced inhibition of DNA synthesis and proliferation in A375 cells (34); (ii) putrescine, a physiological product of ODC reaction and precursor of polyamines, can overcome most when high doses of IL-1 are used (34, 40) and all when low doses of IL-1 are used (40) of IL-1-induced growth inhibition; (iii) in melanoma cells whose ODC activities cannot be down-regulated by IL-1, their growth also cannot be suppressed by IL-1 (40); (iv) treatment of synchronously cultured melanoma cells with IL-1 delays progression from G1/G0 to S, retards progression through G2/M of the first cell cycle, and blocks progression from G0/G1 to S of the second cell cycle (36). This consequence is compatible with IL-1-induced ODC activity down-regulation because it has been reported that ODC activity begins to be blocked after IL-1 was added (36).

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affect ODC mRNA level in C2-1 cells. The results that rhIL-1α did not have any obvious influence on the ODC mRNA level of C2-1 cells (Fig. 3) indicate that IL-1 does not act on the transcription and the stability of ODC mRNA in C2-1 cells. On the other hand, IL-1 decreased ODC protein level of C2-1 cells in a dose- and time-dependent manner (Fig. 4) similar to IL-1-induced ODC activity down-regulation. Furthermore, rhIL-1α treatment induced the production of ODC activity inhibitor(s) in C2-1 cells (Table I). These data led us to propose that both IL-1-induced ODC protein down-regulation and ODC inhibitor(s) accounted for IL-1-induced ODC activity down-regulation.

ODC protein level is regulated at two major sites, i.e. translation and degradation (50–51, 59–62). Theoretically, either decreased translation or increased degradation can result in the down-regulation of ODC protein level. The fact that in IL-1-treated C2-1 melanoma cells, down-regulation of ODC protein was accompanied by the production of ODC inhibitor(s) allowed us to investigate the effect of IL-1 on the expression of AZ, a small intracellular protein known to act as both a specific ODC inhibitor and a promotor for the degradation of ODC protein (50–54, 63). Treatment with rhIL-1α up-modulated AZ mRNA level in a dose- and time-dependent manner (Fig. 5). More important is that rhIL-1α-induced AZ mRNA up-modulation occurred at as early as 6 h after IL-1 addition (Fig. 5B), which preceded the IL-1-induced down-regulation of ODC protein (Fig. 4B) and enzyme activity (Fig. 2B). The transcription of the AZ gene was further shown to be activated by IL-1 treatment (Fig. 6), whereas rhIL-1α treatment had no effect on the stability of AZ mRNA (data not shown). These results suggest that in C2-1 cells, IL-1 probably exerts its growth inhibitory effect by up-modulating AZ expression, which in turn down-regulate ODC activity by simultaneously inhibiting ODC activity and promoting ODC protein degradation. These results also explain why IL-1-induced ODC protein down-regulation was not parallel with IL-1-induced ODC activity down-regulation, because ELISA perhaps measured both free ODC and AZ-bound ODC (without enzymatic activity) molecules that had not been degraded. The importance of IL-1-induced AZ expression in IL-1-induced ODC activity down-regulation is further supported by the fact that antisense AZ RNA expression blocked to a similar extent (about 70%) the IL-1-induced ODC activity down-regulation (Fig. 7A) and growth inhibition (Fig. 7B).

To confirm the role of AZ in IL-1-induced ODC activity down-regulation, another approach would be the direct measurement of AZ protein level in untreated and IL-1-treated cells by methodologies such as Western blotting. Unfortunately, antibodies against human AZ are not available for us, and it is reported that the amount of AZ is quite low (51, 63). How did IL-1-treated C2-1 cell lysate inhibit the ODC activity of untreated C2-1 cell lysate (Table I)? There may be excess AZ in the untreated cell lysate most of the remaining apparent ODC activity was not inhibited by treatment with DFMO, suggesting that there was no ODC activity in the cell lysate. Probably up-modulated AZ protein from the treated cell lysate inhibited ODC activity by binding ODC.

The effects of other cytokines capable of inhibiting the growth of human melanoma cells (34–35, 55–57) on the AZ mRNA levels were also examined, and they all failed to down-modulate AZ mRNA expression in C2-1 cells (Fig. 8). Tumor necrosis factor and interferon has been reported to cause growth inhibition of human melanoma cells by suppressing c-myc expression (58), whereas IL-1 does not inhibit c-myc expression in A375 melanoma cells (38); it is also reported that a number of mutant melanoma cell lines resistant to both IL-6 and oncostatin M remained sensitive to IL-1 (57), and two IL-1-resistant A375 cell lines were still sensitive to IL-6 and oncostatin M (40). These evidences combined with the results of Fig. 8 indicate that IL-1-induced AZ mRNA up-modulation is quite unique to and important for the anti-proliferative effect of IL-1 on melanoma cells. Another line of evidence to support the involvement of AZ mRNA up-modulation in IL-1-induced growth inhibition is that in two IL-1-resistant clones derived from A375–6 cells (56), IL-1 failed to induce AZ mRNA up-modulation as well as ODC activity down-regulation.2

The observation that IL-1 up-modulated AZ expression by accelerating AZ gene transcription (Fig. 6) is of interest. So far as we know, there is no report showing that any factors can influence AZ gene transcription. Polyamines are important intracellular modulator for AZ but have no influence on AZ mRNA level (51, 63). How IL-1 activates the transcription of AZ gene in C2-1 melanoma cells needs further studies. Nevertheless, the results of the present study not only confirm our previous proposition that down-regulation of ODC activity is an essential step in the IL-1-induced growth inhibition in melanoma cells but also demonstrate that IL-1-induced ODC activity down-regulation is the result of IL-1-induced AZ up-modulation. Furthermore, the present study also suggests for the first time that AZ gene expression may have a novel regulatory role in controlling cell growth.

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