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Antiproliferative Effect of IL-1 Is Mediated by p38 Mitogen-Activated Protein Kinase in Human Melanoma Cell A375[1]

Saotomo Itoh,* Takayuki Hattori,* Hidetoshi Hayashi,* Yukiko Mizutani,* Makoto Todo,* Takemasa Takii,* De Yang,* John C. Lee,† Senya Matsufuji,‡ Yasuko Murakami,‡ Taku Chiba,* and Kikuo Onozaki[2]*

The role of p38 mitogen-activated protein kinase (MAPK) in IL-1-induced growth inhibition was investigated using IL-1-sensitive human melanoma A375-C2-1 cells and IL-1-resistant A375-R8 cells. In both cells, p38 MAPK was activated by IL-1. A selective inhibitor for p38 MAPK, SB203580, almost completely recovered the IL-1-induced growth inhibition in A375-C2-1 cells. IL-1-induced IL-6 production was also suppressed by SB203580. However, the reversal effect of SB203580 was not due to the suppression of IL-6 production because the SB203580 effect was still observed in the presence of exogenous IL-6. Down-regulation of ornithine decarboxylase (ODC) activity as well as its protein level has been shown to be essential for IL-1-induced growth inhibition. SB203580 also reversed the IL-1-induced down-regulation of ODC activity and intracellular polyamine levels without affecting ODC mRNA levels in A375-C2-1 cells. In IL-1-resistant R8 cells, however, IL-1 only slightly suppressed ODC activity. In A375-C2-1 cells, the mRNA expression level of antizyme (AZ), a regulatory factor of ODC activity, has been shown to be up-regulated by IL-1. IL-1-induced up-regulation of AZ mRNA level was not affected by SB203580. These findings demonstrate that p38 MAPK plays an important role in IL-1-induced growth inhibition in A375 cells through down-regulating ODC activity without affecting the level of ODC mRNA and AZ mRNA. In IL-1-resistant A375-R8 cells, IL-1 signaling pathway is deficient between p38 MAPK activation and down-regulation of ODC activity. ***The Journal of Immunology*****, 1999, 162: 7434–7440.**

Interleukin 1 exhibits pleiotropic effects in host reactions, including immune defense, inflammation, and homeostasis (1). At the cellular level, IL-1 affects the functions of many cell types that lead to stimulation/inhibition of cell proliferation, differentiation, and apoptosis. Regarding the cell growth, IL-1 acts either as a growth stimulator for thymocytes (2), B cells (3), and fibroblasts (4), or as an inhibitor for a variety of cells such as pancreatic Langerhans cells (5), endothelial cells (6), breast carcinoma cells, and myeloid leukemia cells (7).

Proliferation of the human melanoma cell line, A375-6 is inhibited by IL-1 (8). In this process, IL-6 production was up-regulated (9), ornithine decarboxylase (ODC)[3] activity was down-regulated (10), and cell cycle progression was arrested at G_0/G_1 phase (11). IL-1-induced growth inhibition was partly recovered by the addition of anti-IL-6 Ab (9) or putrescine (10), a polyamine generated by ODC. Therefore, up-regulation of IL-6 production and down-regulation of ODC activity contribute to the IL-1-induced growth inhibition.

Polyamines (putrescine, spermidine, spermine) are thought to be essential for cell proliferation (12). Intracellular polyamines are synthesized from one of the basic amino acids, L-ornithine. ODC catalyzes the decarboxylation of ornithine to diamine, putrescine. Putrescine is converted to more polycationic polyamines, spermidine and spermine by spermidine synthase and spermine synthase, respectively. ODC is a rate limiting enzyme in the polyamine synthesis (13). The regulation of ODC activity is tightly associated with cell proliferation because ODC specific inhibitor α-difluoromethyl ornithine (DFMO) strongly inhibits cell growth (14), and ODC activity is up-regulated in rapidly growing tissues (15).

We have recently established a highly IL-1-sensitive transfectant A375-C2-1 by transfecting type I IL-1R expression plasmid to A375-5 cells that do not respond to IL-1 because they express no detectable IL-1R (16). Using this transfectant, we reported that IL-1 down-regulates activity and protein level of ODC and up-regulates antizyme (AZ) mRNA, an inhibitor of ODC activity and enhancer for ODC degradation (17). As the down-regulation of ODC activity and growth inhibition by IL-1 were suppressed by transfecting AZ antisense expression plasmid, AZ was revealed to be involved in these processes (18).

Various pathways and factors have been reported to be involved in IL-1 signaling, including Ca^{2+} influx, protein kinase C, cAMP and protein kinase A, sphingomyelinase and ceramide(s), reactive oxygen species, and mitogen-activated protein (MAP) kinases (1). However, we were unable to obtain solid evidences that any of these reported IL-1 signaling pathways were responsible for IL-1 antiproliferative effect.

Recently, two members of new MAP kinases (MAPK), the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase

*Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho, Nagoya, Japan; †SmithKline Beecham, King of Prussia, PA 19406; and ‡Department of Biochemistry II, Jikei University of Medicine, Minato-ku, Tokyo, Japan

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[2] Address correspondence and reprint requests to Dr. Kikuo Onozaki, Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho, Nagoya 467-8603, Japan. E-mail address: konozaki@phar.nagoya-cu.ac.jp

[3] Abbreviations used in this paper: ODC, ornithine decarboxylase; DFMO, α-difluoromethyl ornithine; AZ, antizyme; MAP, mitogen-activated protein; Erk, extracellular signal-regulated kinase; MAPK, MAP kinase; JNK, c-Jun N-terminal kinase; MAPKAPK, MAPK-activated protein kinase; CHOP, C/EBP-homologous protein.

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(JNK) and reactivating kinase (RK)/p38 MAPK, have been reported (19). These MAPKs form a MAPK superfamily together with classical MAPK, extracellular signal-regulated kinase (Erk)-1 and 2. Similar to classical MAPK Erk-1 and -2, these new MAPK also form MAPK signal cascades. Cellular stress, such as osmotic, chemical, heat shock, and cytokines, such as IL-1 and TNF-α, evoke activation of these MAPKs (20–22). These activation are mediated by corresponding upstream MAPK kinase. Erk-1 and -2 are phosphorylated and activated by MEK-1 and -2 (MAP/ERK kinase). p38 MAPK is activated by MAP kinase kinase (MKK)-3 (23) and -6 (24), and SAPK is activated by MKK-4 (23) and MKK-7 (25). Two MAP kinases transduce signal by phosphorylating their substrates, including other kinases and transcription factors (26). SAPK phosphorylates c-Jun and ATF-2 and subsequently increases their transcriptional activity. p38 MAPK phosphorylates MAPK-activated protein kinase (MAPKAPK)-2 and -3, and then these MAPKAPKs phosphorylate their substrate proteins, small heat shock proteins (HSP) 25 and 27 (27). p38 MAPK also phosphorylates transcription factor CHOP (C/EBP-homologous protein) and enhances its transcriptional activity (28). Recently, it has been shown that p38 MAPK activity is inhibited by a class of pyridinyl-imidazole compound, exemplified by SB203580, a selective p38 MAPK inhibitor (29, 30). Therefore, this compound is quite useful for investigation of the role of p38 MAPK.

In this paper, we investigated whether p38 MAPK plays a role in IL-1-induced growth inhibition of A375 melanoma cells. Using the inhibitor SB203580, we demonstrated that p38 MAPK is involved in IL-1-induced down-regulation of ODC activity and growth inhibition without affecting the level of ODC mRNA and AZ mRNA. In IL-1-resistant subline A375-R8, neither growth inhibition nor down-regulation of ODC activity were induced by IL-1 irrespective of p38 MAPK activation; therefore, IL-1 signaling pathway between p38 MAPK activation and ODC activity down-regulation is deficient in this resistant cell.

Materials and Methods

Reagents

RPMI 1640 medium was purchased from Sigma (St. Louis, MO); FBS was from JRH Biosciences (Lenexa, KS); and human recombinant IL-1α (2×10^7 U/mg) was provided by Dr. M. Yamada (Dainippon Pharmaceutical, Osaka, Japan). Human recombinant IL-6 was a gift from Dr. Y. Akiyama (Ajinomoto, Yokohama, Japan). Ab against rabbit MAPKAPK-2 (sheep polyclonal IgG) and a substrate peptide (KKLNRTLSVA) for MAPKAPK-2 assay were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-p44/42 MAP kinase polyclonal Ab and anti-phospho p44/42 MAPK mAb were purchased from New England Biolabs (Beverly, MA). Anti-HA mAb (12CA5) was from Boehringer Mannheim (Mannheim, Germany). HRP-conjugated goat anti-rabbit IgG and HRP-conjugated rabbit anti-mouse IgG were obtained from Amersham (Aylesbury, U.K.). pGEX-c-jun(1–71) and pSRα-HA-JNK1 were obtained from Dr. T. Sudo (Institute of Physical and Chemical Research (RIKEN), Wako, Japan). GST-c-Jun(1–71) was expressed as a GST fusion protein in *Escherichia coli* and purified by reduced glutathione (GSH)-Sepharose (Pharmacia Biotech, Piscataway, NJ).

Cell culture

A human melanoma cell line A375 was obtained from Dr. R. Ruddon (National Cancer Institute, Bethesda, MD). By limiting dilution, IL-1-sensitive clone A375-6 and insensitive clone A375-5 were obtained. One of the IL-1-resistant subclones, A375-R8 was obtained by limiting dilution of A375-6 cells that had acquired resistance to IL-1 after routine passage for 3 mo. A375-R8 cells express comparable level of type I IL-1R mRNA as A375-6 (31). A375-5 cells are resistant to IL-1 because the cells express undetectable level of IL-1R (16). A375-C2-1, a highly IL-1-sensitive clone, was obtained by transfection with human type I IL-1R expression plasmid to A375-5 cells. These cells were cultured in RPMI 1640, 100 U/ml penicillin G, 100 μg/ml streptomycin, 15 mM HEPES, and 5% heat-inactivated FBS at 37°C.

In vitro kinase assay for MAPKAPK-2

Preparation of cell lysates, immunoprecipitation, and in vitro kinase assay for MAPKAPK-2 (32) were performed according to the protocol of the supplier with slight modifications. A375 cells were pretreated with SB203580 and then treated with IL-1α as described in the figure legends. The cells were washed twice with ice-cold PBS and solubilized on ice for 15 min with buffer A (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5 mM sodium orthovanadate, 0.1% 2-ME, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 50 mM NaF) and centrifuged at 18,000 $\times$ *g* for 5 min at 4°C. The supernatants were immunoprecipitated by incubation with anti-MAPKAPK-2 Ab-bound protein G-Sepharose beads for 2 h at 4°C. The beads were washed with buffer A supplemented with 0.5 M NaCl and with assay dilution buffer (ADB: 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT). To 50 μl of the protein G-Sepharose/immunocomplex, 10 μl of [γ-^{32}P]ATP(10 μCi; Amersham) diluted with ADB containing 75 mM $MgCl_2$, 5 μl of substrate peptide (125 μmol), and 25 μl of ADB were added. After incubation for 30 min at 30°C, a 25 μl aliquot was recovered and spotted onto a P81 paper disc (Whatman, Maidstone, U.K.) and washed three times with 0.75% phosphoric acid. Once washed with acetone and dried, the radioactivity bound to the disc was counted in a scintillation counter (LSC-5500; Aloka, Tokyo, Japan).

Assay for JNK activity

A375-C2-1 cells in 6-well plates (60–70% confluent) were transiently transfected with 0.4 μg of pSRα-HA-JNK1 using Effectene Transfection Reagent (Qiagen, Hilden, Germany). After 48 h, the cells were collected and the lysates were prepared as described for the MAPKAPK-2 assay. HA-JNK1 was immunoprecipitated with anti-HA mAb (12CA5) and protein A-Sepharose. The immune complex was incubated for 15 min at 30°C with [γ-^{32}P]ATP (2 μCi; Amersham) and 10 μg of GST-c-Jun (1–79) as a substrate. The reaction was terminated with Laemmli sample buffer, and the products were resolved by 14% SDS-PAGE. Substrate phosphorylation was visualized with a Bioimage analyzer (BAS-2500; Fuji, Tokyo, Japan).

Activation of Erk1/2

A375-C2-1 cells in 6-well plates (60–70% confluent) were incubated in the presence or absence of 1000 U/ml of IL-1α for 10 min, and the lysates were prepared as described for the MAPKAPK-2 assay. Lysates were electrophoresed with 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The phosphorylated p44/42 MAPK was detected by Western blot analysis with phospho-p44/42 MAPK and HRP-conjugated rabbit anti-mouse IgG. For detection, an enhanced chemiluminescence (ECL) kit (Amersham) and a Lumino Image Analyzer, LAS-1000 (Fuji), were used.

Assays for cell proliferation

Cells were detached from the culture dish with 0.02% EDTA-PBS. After washing the cells with culture medium, 100 μl of cell suspension (2×10^4 cells/ml) were added to each well of 96-well flat-bottom microtiter plate (Falcon, Lincoln, NJ). After 24-h cultivation, 100 μl of medium containing varying concentrations of cytokines or 10 μM of SB203580 were added. Then the cells were cultured for another 72 h. Proliferation of the cells was determined by staining with crystal violet (33). After solubilization of the dye-stained cells with 0.1% SDS, the dye uptake was calculated by measuring the absorbance at 595 nm by a microplate autoreader (Bio-Rad, Richmond, CA).

Assay for IL-6 activity

Biological activity of IL-6 was measured by its proliferative action on the IL-6-dependent murine hybridoma clone, MH60.BSF2 (34), provided by Dr. T. Hirano (Osaka University, Osaka, Japan). Cells were cultured in wells of a flat-bottom microtiter plate at 37°C in 5% CO_2 in air. After 3 days of culture, cell proliferation activity was assessed by the MTT method (35). After solubilizing the formazan with 20% SDS and 50% dimethyl formamide in water, the absorbance at 595 nm was measured by an ELISA autoreader. IL-6 activity was expressed as the equivalent amount of recombinant human IL-6.

Assays for ODC activity

Cells were seeded subconfluently in 100-mm dishes and treated with or without IL-1α (1000 U/ml) for 48 h in the absence or presence of SB203580 (10 μM). After incubation, cells were collected by trypsinization and washed twice with ice-cold TED buffer (25 mM Tris, 0.15 mM

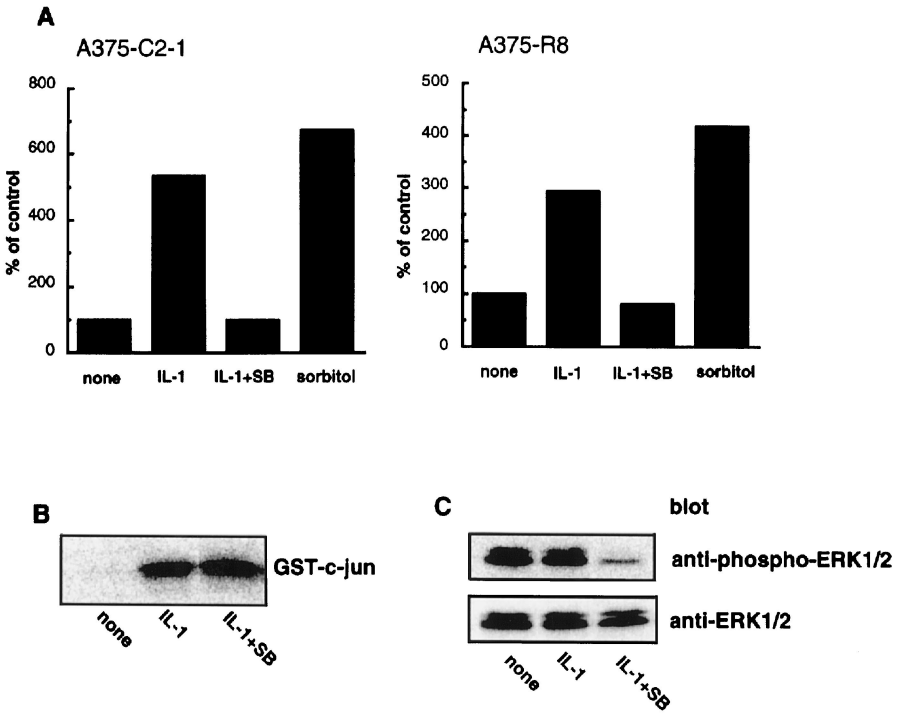

FIGURE 1. Effect of IL-1 on MAPK activities in A375 cells. *A*, Activation of MAPKAPK-2. Extracts were prepared from A375-C2-1 or A375-R8 cells that were treated with IL-1α (1000 U/ml) or sorbitol (400 mM) at 37°C for 15 min in the presence or absence of 10 μM of SB203580. SB203580 was added for 1 h prior to the treatment with these cytokines. Immune complex kinase assay was performed as described in *Materials and Methods*. Representative data of two independent experiments with similar results are shown. *B*, Activation of JNK. pSRα-HA-JNK1-transfected A375-C2-1 cells were pretreated with or without SB203580 at 10 μM for 1 h. HA-JNK1 in the cell treated with or without 1000 U/ml IL-1α for 15 min was immunoprecipitated, and an immune complex kinase assay was performed for JNK using GST-c-Jun(1–79) as a substrate. *C*, Activation of Erk1/2. A375-C2-1 cells were pretreated with or without SB203580 at 10 μM for 1 h prior to IL-1α treatment (1000 U/ml for 10 min) and the extracts were subjected to immunoblotting with anti-phospho-p44/42 MAPK (*upper*) or anti-p44/42 MAPK Abs (*lower*).

EDTA, 2.5 mM DTT (pH 7.5)) containing 0.15 M NaCl. The cells were resuspended with TED buffer and sonicated for 30 s on ice by a handy sonicator (Tomy Seiko, Tokyo, Japan). Protein content of the supernatants was determined by protein assay kit (Bio-Rad). ODC activity was measured by the method of Seely and Pegg (36) with minor modifications. Briefly, the enzyme reaction mixture consisting of 100 μl of TED buffer, 25 μl of 0.8 mM pyridoxal phosphate, 15 μl of 8 mM L-ornithine, and 10 μl of DL-[1-^{14}C]ornithine was prepared. After the addition of 100 μl of TED 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 [^{14}C]CO_2 (dpm/mg of protein), which was collected on a 4-cm^2 filter paper soaked with 0.5 N NaOH and measured with a fluid scintillation counter.

Assay for intracellular polyamine concentration

Cells were seeded subconfluently in 100-mm dishes in 20 ml culture medium and treated with or without IL-1α (1000 U/ml) for 48 h in the absence or presence of SB203580 (10 μM). Both detached and attached cells were collected and subjected to polyamine determination by the method of Seiler (37) with minor modifications. Cells were washed twice with ice-cold PBS and lysed with 0.2% of Triton-X 100. After determining the protein content, the lysates containing equal amount of proteins were transferred to test tube and deproteinized by 0.4 M $HClO_4$ (final concentration). The supernatants were subjected to dancylation by dancyl chloride. Dancyl polyamines were separated by TLC and visualized by transilluminator and the amounts of polyamines were determined by a Bioimage analyzer (BAS2000; Fuji).

RNA extraction and Northern hybridization

Monolayer subconfluent cultures of A375 cells were treated with IL-1α in the absence or presence of SB203580 as indicated in the figure legends. Total RNA was extracted from cells according to the method of Chomczynski and Sacchi (38). After size fractionation on an agarose-formaldehyde gel and transfer to nitrocellulose filter, the specific mRNA on the filter was detected by hybridization with a ^{32}P-labeled cDNA probe at 42°C for 18 h in hybridization buffer containing 50% formamide, 5× SSPE (1× SSPE = 0.15 M NaCl, 10 mM NaH_2PO_4, 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: 1) a 551-bp fragment corresponding to bases 186–726 of human AZ cDNA; 2) a 611-bp cDNA fragment corresponding to bases 2–612 of human ODC cDNA; and 3) a *Pst*I-digested 1300-bp fragment of human GAPDH cDNA. These probes were labeled by random priming (Multi Prime DNA labeling kit; Amersham). After hybridization, filters were washed for 5 min with 2× SSC/0.1% SDS (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)) at room temperature and washed two times for 30 min with 0.2× SSC/0.1% SDS at 65°C. Filters were autoradiographed using a Bioimage analyzer.

Results

IL-1 increases p38 MAPK activity in A375 cells

First, we examined whether stress-inducible p38 MAPK is activated in A375 human melanoma cells. As p38 MAPK is reported to phosphorylate and activate MAPKAPK-2 (27), we performed the immune complex kinase assay for MAPKAPK-2 to determine p38 MAPK activity. As shown in Fig. 1*A*, MAPKAPK-2 activity was markedly increased by IL-1α (1000 U/ml) in IL-1-sensitive A375-C2-1 cells. Treatment of the cells with 10 μM SB203580, a selective inhibitor for p38 MAPK, resulted in complete inhibition of MAPKAPK-2 activity. Activation of MAPKAPK-2 by IL-1α was also observed in the IL-1-resistant A375 clone A375-R8, and its activation was inhibited by SB203580, as well (Fig. 1*A*). Sorbitol, a hyperosmotic stress signal, also activated the kinase activity in both IL-1-sensitive and IL-1-resistant cells. The effect of IL-1 on the activities of other MAPKs, JNK and Erk1/2, was also examined in A375-C2-1 cells. The activity of JNK was augmented

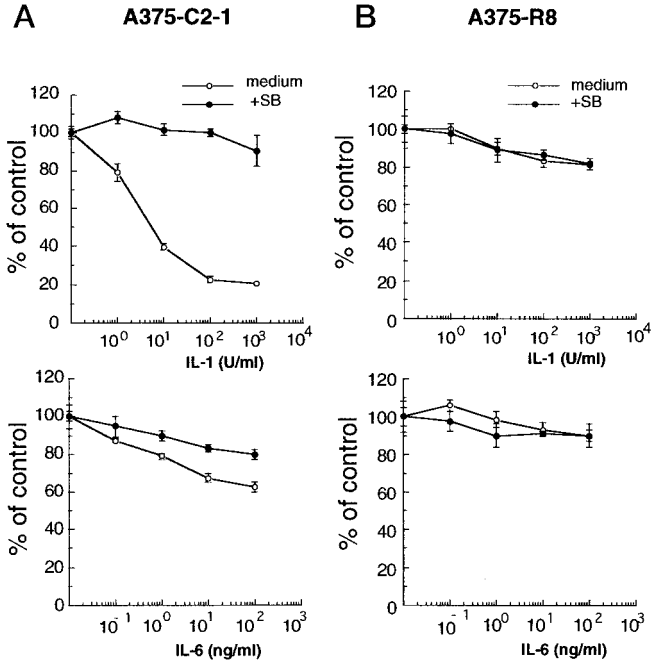

FIGURE 2. Effect of SB203580 on the growth inhibition induced by IL-1α and IL-6 on A375 cells. A375-C2-1 (*A*) or R8 (*B*) cells (2.0×10^3 cells/well) were cultured for 3 days with or without varying doses of IL-1α and IL-6 in the presence or absence of 10 μM of SB203580. After culture, cells proliferation was determined by staining with crystal violet. The cell proliferation in medium or SB203580 alone was used as 100%. Data for the mean $\pm$ SD based on triplicate cultures are shown. The data shown are representative of three independent experiments.

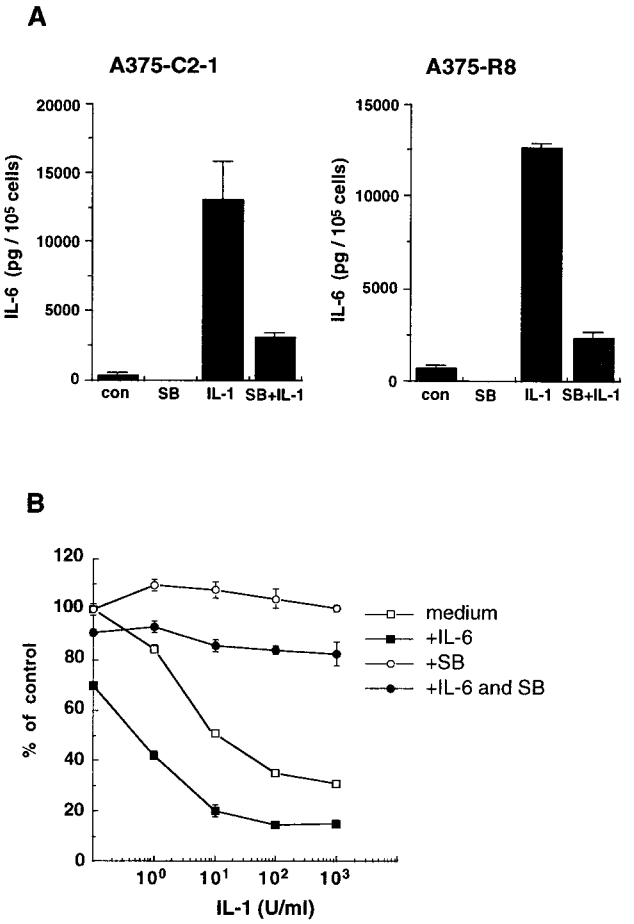

FIGURE 3. Suppression of IL-6 production from A375-C2-1 cells by SB203580 does not contribute to the reversing effect of SB203580. *A*, Effect of SB203580 on IL-6 production from A375 cells. A375-C2-1 or R8 cells (10^5 cells/ml) were treated without or with IL-1α (100 U/ml) in the presence or absence of 10 μM of SB203580, and supernatants were obtained after 24-h culture. After dialysis, IL-6 activity was determined by MH60 cell proliferation assay. Data for the mean $\pm$ SD based on triplicate cultures are shown. The data shown are representative of two independent experiments with similar results. *B*, Effect of SB203580 on the growth of A375-C2-1 cells in the presence of exogenous IL-6. A375-C2-1 cells (2.0×10^3cells/well) were cultured for 3 days with or without varying doses of IL-1α in the presence or absence of 10 μM of SB203580 and IL-6 (100 ng/ml). After culture, cell proliferation was determined by staining with crystal violet. The cell proliferation in medium or SB203580 alone was used as 100%. Data for the mean $\pm$ SD based on triplicate cultures are shown. The data shown are representative of three independent experiments.

by IL-1, and its activation was not affected by SB203580 (Fig. 1*B*). In contrast, Erk1/2 was constitutively activated as revealed by the phosphorylation and its activity was not augmented by IL-1 (Fig. 1*C*). SB203580 at 10 μM inhibited the activity with or without IL-1 (data not shown).

SB203580 reverses IL-1- and IL-6-induced growth inhibition in A375 cells

Next, we examined the effect of SB203580 on IL-1-induced growth inhibition in A375-C2-1 cells. As shown in Fig. 2, IL-1 inhibited the proliferation of A375-C2-1 cells, and SB203580 (10 μM) reversed growth inhibition almost completely. IL-6 also inhibited the growth of C2-1 cells, but SB203580 had a lesser effect on IL-6-mediated growth inhibition. In contrast, IL-1 and IL-6 exhibited no or marginal inhibitory effect on the proliferation of A375-R8 cells. These results suggest that p38 MAPK is critically involved in IL-1 signaling leading to growth inhibition, while it partly mediates the growth inhibitory effect of IL-6 in A375-C2-1 cells.

SB203580 inhibits IL-6 production by IL-1 treatment

In IL-1-sensitive A375-6 cells, IL-1-induced IL-6 contributes to the antiproliferative effect of IL-1, as IL-1 antiproliferative effect was partly neutralized by anti-IL-6 Ab (9). Since p38 MAPK regulates cytokine production including IL-6 (39), it is conceivable that SB203580 might have reversed IL-1-induced growth inhibition through suppression of IL-6 production. As shown in Fig. 3*A*, SB203580 (10 μM) inhibited IL-6 production in IL-1-treated A375-C2-1 cells. We next examined the effect of SB203580 on IL-1-induced growth inhibition in the presence of excess IL-6 (100 ng/ml) (Fig. 3*B*). SB203580 was effective even in the presence of IL-6, indicating that the effect of SB203580 was not due to suppression of IL-6 production. We have reported that A375-R8 cells produce IL-1α constitutively (31); therefore, the cells also produce IL-6 constitutively as a result of autocrine stimulation of IL-1α, and IL-6 production is further enhanced by exogenous IL-1. Both constitutive and inducible IL-6 production from R8 cells were also inhibited by SB203580 (Fig. 3*B*). These results suggest that IL-1-induced IL-6 production was mediated by p38 MAPK and this IL-1 signaling pathway is intact in R8 cells.

SB203580 reverses IL-1-induced down-regulation of ODC activity and intracellular polyamine level

We have previously reported that down-regulation of ODC activity is essential for IL-1-induced growth inhibition (10, 16). Therefore,

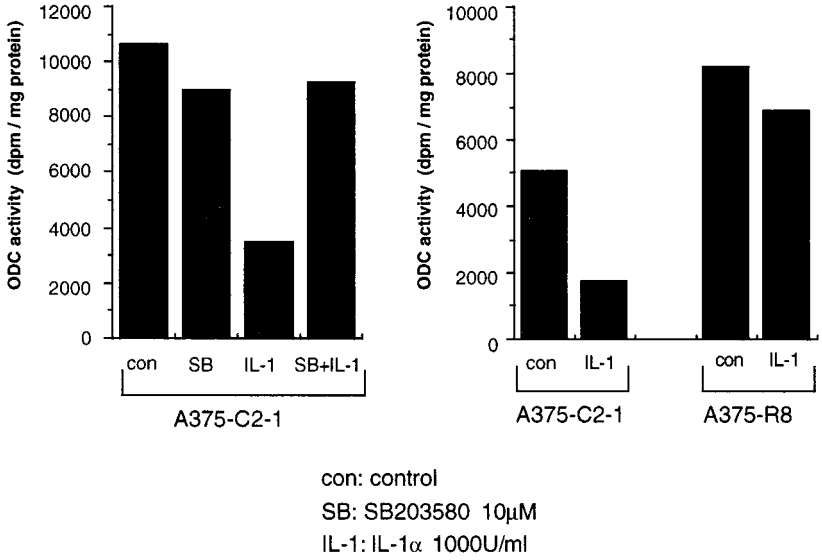

FIGURE 4. Effect of SB203580 on IL-1-induced down-regulation of ODC activity in A375 cells. Subconfluent cultures of A375-C2-1 or A375-R8 cells were treated with IL-1α (1000 U/ml) for 48 h in the presence or absence of 10 μM of SB203580. SB203580 was added for 1 h prior to the treatment with IL-1α. After incubation, ODC activity was determined as described in *Materials and Methods*. The data shown are representative of three independent experiments.

we examined whether SB203580 reversed IL-1-induced down-regulation of ODC activity. As shown in Fig. 4, SB203580 significantly normalized ODC activity induced by IL-1 in IL-1-sensitive A375-C2-1 cells. The slight decrease of ODC activity in SB203580-treated cells was not significant. IL-1 decreased intracellular polyamine concentration, and SB203580 restored intracellular polyamine level in A375-C2-1 cells (Fig. 5). On the other hand, IL-1 only slightly down-regulated ODC activity in IL-1-resistant A375-R8 cells (Fig. 4), and SB203580 did not affect the ODC activity of A375-R8 cells (data not shown). These results suggest that p38 MAPK activation lay upstream of ODC and the activation of p38 MAPK leads to down-regulation of ODC activity. It can be concluded that this pathway is deficient in IL-1-resistant A375-R8 cells.

SB203580 does not affect ODC and AZ mRNA level

To investigate the mechanism of IL-1-induced down-regulation of ODC activity, we examined the effect of IL-1 and SB203580 on ODC mRNA expression. As shown in Fig. 6*A*, IL-1 and SB203580, either alone or in combination, did not affect the expression level of ODC mRNA in A375-C2-1 cells. We have reported that IL-1 up-regulated mRNA expression level of AZ in A375-C2-1 cells (18). As shown in Fig. 6*B*, IL-1-induced up-regulation of AZ mRNA was not affected by SB203580 treatment.

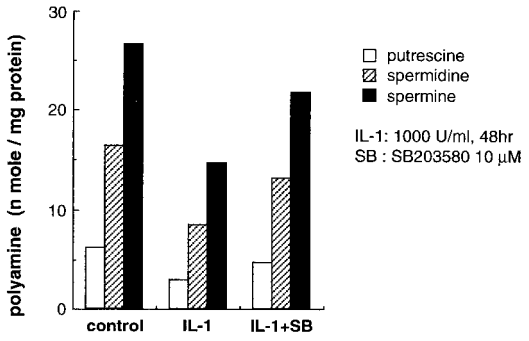

FIGURE 5. Effect of SB203580 on IL-1-induced down-regulation of intracellular polyamine levels. A375-C2-1 cells were treated with IL-1α (1000 U/ml) in the presence of absence of 10 μM of SB203580. SB203580 was added for 1 h prior to the treatment with IL-1α. After 48 h, cells were detached and lysed with 0.2% of Triton X-100. Polyamine concentration was determined as described in *Materials and Methods*. The data shown are representative of three independent experiments.

Discussion

p38 MAPK is known to be activated by several stressful stimuli, such as UV, heat shock, and osmotic shock. p38 MAPK is also activated by proinflammatory cytokines, IL-1 and TNF-α, and is involved in signal transduction pathways. In this study, we demonstrate that p38 MAPK plays an important role in IL-1-induced growth inhibition in A375 cells. In IL-1-sensitive A375-C2-1 cells, IL-1 activated p38 MAPK activity. SB203580, a selective p38 MAPK inhibitor, inhibited the IL-1-induced activation of p38 MAPK. Another MAPK, JNK was also activated by IL-1; however, its activation was not affected by SB203580. In contrast, Erk was constitutively activated, and its activity was not augmented by IL-1. Unexpectedly, SB203580 inhibited the activation of Erk. However, it is unlikely that Erk is responsible for the antiproliferative effect of IL-1 because its activation was not affected by IL-1. SB203580 also reversed IL-1-induced growth inhibition as well as down-regulation of ODC activity and intracellular polyamine levels. It has been shown that the down-regulation of ODC activity is essential for IL-1-induced growth inhibition (10, 16). Similar recovery of IL-1-induced growth inhibition and ODC activity down-regulation was observed by the addition of SB203580 in another IL-1-sensitive A375 cell, A375-6 (data not shown). Taken together, p38 MAPK appeared to mediate the antiproliferative effect of IL-1 through down-regulation of ODC activity in IL-1-sensitive A375 cells.

It remains to be elucidated how p38 MAPK regulates ODC activity. ODC is regulated by AZ, which is known to be induced by polyamines, inhibits ODC activity by forming a complex with ODC, and enhances the degradation of ODC through the 26S proteasome (17). We have previously reported that AZ is responsible for IL-1-induced growth inhibition in A375-C2-1 cells since IL-1 up-regulated AZ mRNA in the cells. Moreover, transfection with

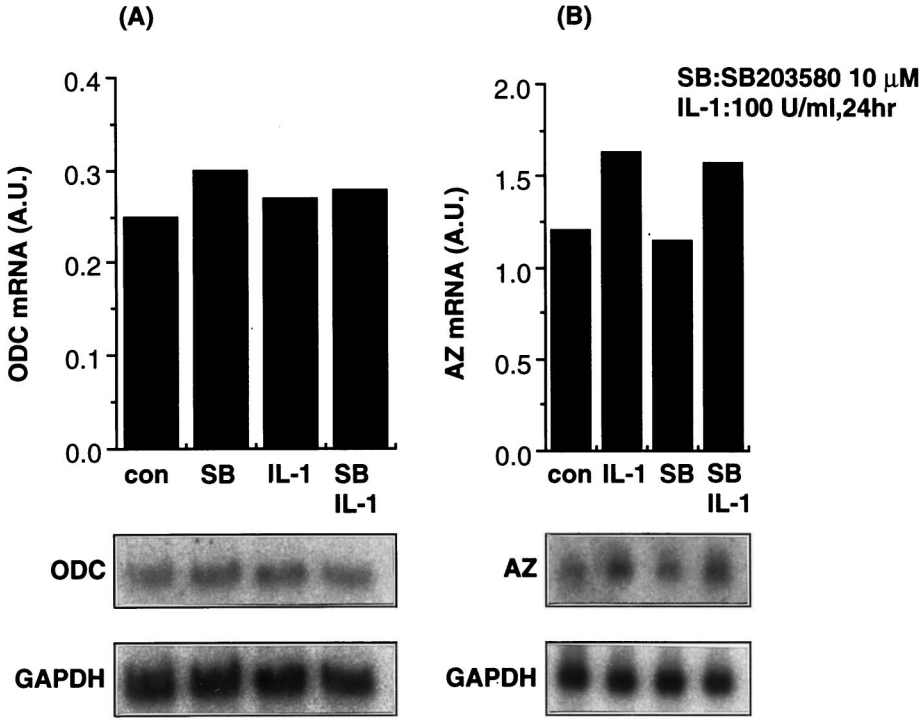

FIGURE 6. SB203580 does not affect the expression of ODC and AZ mRNA. Total RNA was extracted from A375-C2-1 cells that were treated with IL-1α(100U/ml) in the presence or absence of 10 μM of SB203580 for 24 h. SB203580 was added for 1 h prior to the treatment with IL-1. Northern blot analysis was performed as described in *Materials and Methods.* The data shown are representative of three independent experiments. A.U. (arbitrary unit) represents the data normalized by the intensity of GAPDH mRNA.

AZ antisense RNA expression plasmid reversed IL-1-induced growth inhibition (18). However, while IL-1-induced down-regulation of ODC activity was reversed by SB203580, up-regulation of AZ mRNA by IL-1 was not. In another IL-1-sensitive melanoma cell, A375-6, irrespective of IL-1-induced down-regulation of ODC activity and cell proliferation, AZ mRNA was not up-regulated by IL-1 (data not shown). Therefore, these results suggest that the up-regulation of AZ mRNA is not essential or sufficient for the down-regulation of ODC activity and growth inhibition by IL-1, although it may contribute by augmenting AZ activity. In this regard, A375-C2-1 cells are more sensitive to IL-1 than A375-6 cells.

We have reported that IL-6 comparably inhibited the proliferation of A375-C2-1 and A375-6 cells and that IL-6 induced by IL-1 contributes to IL-1-induced growth inhibition in A375-6 cells because anti-IL-6 Ab, in part, prevented IL-1-induced growth inhibition (9). Therefore, it was expected that SB203580 prevented the IL-1 antiproliferative effect in A375-C2-1 cells through the down-regulation of IL-6 production. Indeed, IL-6 production was inhibited by SB203580. However, SB203580 prevented IL-1-induced growth inhibition irrespective of the addition of excess amount of IL-6. Perhaps the IL-1 signal is more potent in A375-C2-1 cells than A375-6 cells, thus the contribution of IL-6 is less in A375-C2-1 cells.

IL-1-sensitive A375 cells often acquire resistance to IL-1 antiproliferative effect during long-term culture. A375-R8 cells are an IL-1-resistant subclone established from A375-6 cells that acquired resistance to IL-1 (31). To elucidate the mechanism for the gain resistance, it is valuable to understanding the mechanism of IL-1-induced growth inhibition in A375 cells. As shown in this study, p38 MAPK is activated by IL-1 in A375-R8 cells; however, ODC activity was not down-regulated by IL-1. In A375-R8 cells, stimulation of IL-6 production by IL-1 was observed similarly to A375-C2-1 cells, and it was suppressed by SB203580. These results indicate that IL-1 signal transduction pathway leading to p38 MAPK activation and IL-6 production is intact in IL-1-resistant cells. However, signaling pathway leading to the down-regulation of ODC activity is impaired. Previously, we have demonstrated that acquired resistance to IL-1 in A375-R8 cells is a recessive phenotype using somatic cell hybridization (39). Thus, factor(s) involved in IL-1 antiproliferative effect is deficient in A375-R8 cells. Taken together, IL-1 signaling pathway leading to growth inhibition is deficient at a step lying somewhere between p38 MAPK activation and down-regulation of ODC activity in A375-R8 cells. This possibility was supported by the observations that DFMO, a specific inhibitor of ODC activity, inhibited proliferation of either A375-C2-1 or A375-R8 cells in a same manner, and the growth inhibition was not reversed by SB203580 (data not shown).

SB203580 was able to recover IL-1-induced growth inhibition almost completely, whereas putrescine, a product of ODC, did so partly. Therefore, the down-regulation of ODC activity may not be the only manner leading to inhibition of cell growth. It is possible that other factor(s) lie downstream from p38 MAPK plays an important role in IL-1-induced growth inhibition. It has been reported that activities of several factors related in cell growth are regulated by p38 MAPK. CHOP, which belongs to the family of C/EBP transcription factors, induces growth inhibition when it is highly expressed (40). Recently CHOP has been reported to be phosphorylated by p38 MAPK and the phosphorylation is implicated in its activation (28). Therefore, CHOP may be one of the candidates involved in IL-1-induced growth inhibition. Another candidate is cyclin D1, an important factor for cell cycle progression, the expression of which is up-regulated by classical MAPK and is down-regulated by p38 MAPK (41). In our preliminary study, however, the mRNA level of cyclin D1 was up-regulated by IL-1. Recent

studies revealed that there are several isoforms in p38 MAPK, α, β, γ, and δ (42). Therefore, it is also interesting to determine the type of isoform involved in this IL-1 signaling.

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References

1. Dinarello, C. A. 1996. Biological basis for interleukin-1 in disease. *Blood 87: 2095.*
2. Gery, I., R. K. Gershon, and B. H. Waksman. 1972. Potentiation of the T-lymphocytes response to mitogens. I. The responding cell. *J. Exp. Med. 136:128.*
3. Freedman, A. S., G. Freeman, J. Whitman, J. Segil, J. Daley, and J. M. Nadler. 1988. Pre-exposure of human B cells to recombinant IL-1 enhances subsequent proliferation. *J. Immunol. 141:3398.*
4. Schmidt, J. A., S. B. Mizel, D. Cohen, and I. Green. 1982. Interleukin 1, a potential regulator of fibroblast proliferation. *J. Immunol. 128:2177.*
5. Reimers, J. I, H. U. Andersen, D. Mauricio, F. Pociot, A. E. Karlsen, J. S. Petersen, T. Mandrup-Poulsen, and J. Nerup. 1996. Strain-dependent differences in sensitivity of rat β cells to interleukin 1β in vitro and in vivo: association with islet nitric oxide synthesis. *Diabetes 45:771.*
6. Maier, J. A., P. Voulalas, D. Roeder, and T. Maciag. 1990. Extension of the life-span of human endothelial cells by an interleukin-1α antisense oligomer. *Science 249:1570.*
7. Onozaki, K., T. Tamatani, T. Hashimoto, and K. Matsushima. 1987. Growth inhibition and augmentation of mouse myeloid leukemic cell line differentiation by interleukin 1. *Cancer Res. 47:2397.*
8. Onozaki, K., K. Matsushima, B. B. Aggarwal, and J. J. Oppenheim. 1985. Human interleukin 1 is a cytocidal factor for several tumor cell lines. *J. Immunol. 135:3962.*
9. Morinaga, Y., H. Suzuki, F. Takatsuki, Y. Akiyama, T. Taniyama, K. Matsushima, and K. Onozaki. 1989. Contribution of IL-6 to the antiproliferative effect of IL-1 and tumor necrosis factor on tumor cell lines. *J. Immunol. 143:3538.*
10. Endo, Y., K. Matsushima, K. Onozaki, and J. J. Oppenheim. 1988. Role of ornithine decarboxylase in the regulation of cell growth by IL-1 and tumor necrosis factor. *J. Immunol. 141:2342.*
11. Morinaga, Y., H. Hayashi, A. Takeuchi, and K. Onozaki. 1990. Antiproliferative effect of interleukin 1 (IL-1) on tumor cells: G_0-G_1 arrest of a human melanoma cell line by IL-1. *Biochem. Biophys. Res. Commun. 173:186.*
12. Tabor, C. W., and H. Tabor. 1984. Polyamines. *Annu. Rev. Biochem. 53:749.*
13. Hayashi, S. 1989. *Ornithine Decarboxylase: Biology, Enzymology and Molecular Genetics*. Pergamon, New York. pp. 35–45.
14. Seidenfeld, J. 1985. Effects of difluoromethylornithine on proliferation, polyamine content and plating efficiency of cultured human carcinoma cells. *Cancer Chemother. Pharmacol. 15:196.*
15. Heby, O., L. J. Marton, C. B. Wilson, and H. M. Martinez. 1975. Polyamines: a high correlation with cell replication. *FEBS Lett. 50:1.*
16. Yang, D., H. Hayashi, Y. Hiyama, T. Takii, and K. Onozaki. 1996. Transfection of human melanoma cells with type I interleukin-1 (IL-1) receptor cDNA rendered them IL-1 responsive and revealed the importance of ODC activity down-regulation in IL-1 induced growth inhibition. *J. Biochem. (Tokyo) 118:802.*
17. Hayashi, S., Y. Murakami, and S. Matsufuji. 1996. Ornithine decarboxylase antizyme: a novel type of regulatory protein. *Trends Biochem. Sci. 21:27.*
18. Yang, D., H. Hayashi, T. Takii, Y. Mizutani, Y. Inukai, and K. Onozaki. 1997. Interleukin-1-induced growth inhibition of human melanoma cells: interleukin-1-induced antizyme expression is responsible for ornithine decarboxylase activity down-regulation. *J. Biol. Chem. 272:3376.*
19. Cano, E., and L. C. Mahadevan. 1995. Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci. 20:117.*
20. Galcheva-Gargova, Z., B. Derijard, I. H. Wu, and R. J. Davis. 1994. An osmosensing signal transduction pathway in mammalian cells. *Science 265:806.*
21. Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett. 1994. The stress-activated protein kinase subfamily of c-*jun* kinases. *Nature 369:156.*
22. Raingeaud, J., S. Gupta, J. S. Rogers, M. Dickens, J. Han, R. J. Ulevitch, and R. J. Davis. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem. 270:7420.*
23. Derijard, B., J. Raingeaud, T. Barrett, I. H. Wu, J. Han, R. J. Ulevitch, and R. J. Davis. 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science 267:682.*
24. Han, J., J. D. Lee, Y. Jiang, Z. Li, L. Feng, and R. J. Ulevitch. 1996. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J. Biol. Chem. 271:2886.*
25. Matsuda, S., T. Moriguchi, S. Koyasu, and E. Nishida. 1998. T lymphocyte activation signals for interleukin-2 production involve activation of MKK6–p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A. *J. Biol. Chem. 273:12378.*
26. Cohen, P. 1997. The search for physiological substances of MAP and SAP kinases in mammalian cells. *Trends Cell Biol. 7:353.*
27. Rouse, J., P. Cohen, S. Trigon, M. Morange, A. Alonso-Llamazares, D. Zamanillo, T. Hunt, and A. R. Nebreda. 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell 78:1027.*
28. Wang, X. Z., and D. Ron. 1996. Stress-induced phosphorylation and activation of the transcription factor CHOP(GADD153) by p38 MAP kinase. *Science 272:1347.*
29. Lee, J. C., J. T. Laydon, P. C. McDonnell, T. F. Gallagher, S. Kumar, D. Green, D. McNulty, M. J. Blumenthal, R. J. Heys, S. W. Landvatter, et al. 1994. A protein kinase is involved in the regulation of inflammatory cytokine biosynthesis. *Nature 372:739.*
30. Cuenda, A., J. Rouse, Y. N. Doza, R. Meier, P. Cohen, T. F. Gallagher, P. R. Young, and J. C. Lee. 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett. 364:229.*
31. Araki, M., T. Yano, H. Hayashi, T. Takii, K. Suzuki, and K. Onozaki. 1994. Resistance to the anti-proliferative effect of IL-1 on human melanoma cell line is associated with endogenous production of IL-1 and IL-6. *Int. J. Cancer 56:275.*
32. Stokoe, D., D. G. Campbell, S. Nakielny, H. Hidaka, S. J. Leevers, C. Marshall, and P. Cohen. 1992. MAPKAP kinase-2: a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J. 11:3985.*
33. Ruff, M. R., and G. E. Gifford. 1980. Purification and physico-chemical characterization of rabbit tumor necrosis factor. *J. Immunol. 125:1671.*
34. Matsuda, T., T. Hirano, and T. Kishimoto. 1988. Establishment of an interleukin 6 (IL-6)/B cell stimulatory factor 2-dependent cell line and preparation of anti-IL-6 monoclonal antibodies. *Eur. J. Immunol. 18:951–956.*
35. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J. Immunol. Methods 65:55.*
36. Seely, J. E., and A. E. Pegg. 1983. Ornithine decarboxylase (mouse kidney). *Methods Enzymol. 94:158.*
37. Seiler, N. 1983. Liquid chromatographic methods for assaying polyamines using prechromatographic derivatization. *Methods Enzymol. 94:10.*
38. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium. *Anal. Biochem. 162:156.*
39. Itoh, S., H. Hayashi, D. Yang, T. Takii, and K. Onozaki. 1997. Acquired resistance to the anti-proliferative effect of interleukin-1 and interleukin-6 is a recessive phenotype in A375 human melanoma cells. *Melanoma Res. 7:455.*
40. Choi, A. M., J. Fargnoli, S. G. Carlson, and N. J. Holbrook. 1992. Cell growth inhibition by prostaglandin A2 results in elevated expression of gadd 153 mRNA. *Exp. Cell Res. 199:85.*
41. Lavoie, J. N., G. L'Allemain, A. Brunet, R. Muller, and J. Pouyssegur. 1996. Cyclin D1 expression is regulated positively by the p42/p44 MAPK and negatively by the p38/HOGMAPK pathway. *J. Biol. Chem. 271:20608.*
42. Jiang, Y., H. Gram, M. Zhao, L. New, J. Gu, L. Feng, F. D. Padova, R. J. Ulevitch, and J. Han. 1997. Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinase, p38δ. *J. Biol. Chem. 272:30122.*