Amurubicinol-induced eotaxin-3 expression in human NCI-H69 small cell lung carcinoma cells

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Abstract. We previously demonstrated the doxorubicin-induced expression of urokinase-type plasminogen activator (uPA), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α in human RC-K8 lymphoma cells and NCI-H69 small cell lung carcinoma cells in which reactive oxygen species might be involved. Amurubicin hydrochloride (AMR), a novel derivative drug of doxorubicin, was recently introduced to clinical practice for treatment of lung cancer in Japan. Therefore, we investigated the effects of AMR on the expression of uPA and chemokines in NCI-H69 cells. AMR and its active form, amurubicinol hydrochloride (AMROH), both induced the expression of uPA, IL-8 and MCP-1 in H69 cells in a dose-dependent manner. When the cultured supernatant obtained from AMR-treated H69 cells was subcutaneously injected into rabbits, migration of a significant number of eosinophils was observed around the injected site. Antigen levels of eotaxin-3, a major migration-factor of eosinophils, were increased in AMROH-treated cells in parallel with the mRNA levels. The induction was observed below the clinically achievable concentration of AMR or AMROH. Thus, the simultaneous induction of uPA, IL-8, MCP-1 and eotaxin-3 may play a role in the pharmacological action of AMR through induction of the interaction between proinflammatory cells and lung carcinoma cells.

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Abbreviations: AMR, amurubicin hydrochloride; AMROH, amurubicinol hydrochloride; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehydes-3-phophate dehydrogenase; IL, interleukin; JNK, c-jun N-terminal protein kinase; MAP, mitogen-activated protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tertrazolium bromide; RANTES, regulated upon activation, normal T-cell expressed and secreted; ROS, reactive oxygen species; SCLC, small cell lung carcinoma; uPA, urokinase-type plasminogen activator

Key words: amurubicin, amurubicinol, eotaxin-3, small cell lung carcinoma cells, urokinase-type plasminogen activator, interleukin-8, monocyte chemoattractant protein-1

Introduction

Doxorubicin induces apoptosis of human malignant cells possibly through generating reactive oxygen species (ROS) in the cells (1). Doxorubicin is the most widely used anticancer agent and is a key drug for the treatment of malignant lymphoma and small cell lung cancer (SCLC) (2,3). We have previously reported doxorubicin-induced urokinase-type plasminogen activator (uPA) expression in two human malignant cell lines, RC-K8 lymphoma cells and NCI-H69 SCLC cells (4). The maximum induction of these factors was observed at 'sublethal' concentrations of doxorubicin where cell-growth was slightly inhibited. Furthermore, we also demonstrated doxorubicin-induced interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) (5) and tumor necrosis factor-α (TNF-α) expressions (6) in human RC-K8 lymphoma cells and NCI-H69 SCLC cells.

Amurubicin hydrochloride (AMR) is a novel, completely synthetic 9-aminoanthracycline derivative. Amurubicinol hydrochloride (AMROH), the C-13 alcohol metabolite of AMR, is the active form of AMR. AMROH was detected as a major metabolite in the tumor tissue of AMR-treated mice (7). This metabolite may be in large part account for the biological activity of AMR *in vivo* also in humans and its anti-tumor activity appeared to be 10 times stronger than that of AMR (8). Achievable plasma levels of AMR and AMROH are 20 μM and 2 μM, respectively, and the half-life of AMR is 1.6 h, and that of AMROH is 6.75 h (7). Both are potent inhibitors of topoisomerase II, and the anti-tumor activity of AMR was shown to be superior to that of doxorubicin (9). uPA, IL-8 and MCP-1 all play significant roles in tumor-growth and invasion because uPA could not only dissolve extracellular matrix but activate IL-8; and IL-8 and MCP-1 are very active chemokines that chemoattract leukocytes and monocytes/macrophages, respectively (10-13). We investigated the effects of AMR and AMROH on the expression of uPA, IL-8 and MCP-1 in H69 cells, and confirmed the induction of uPA, IL-8 and MCP-1 by ELISA,

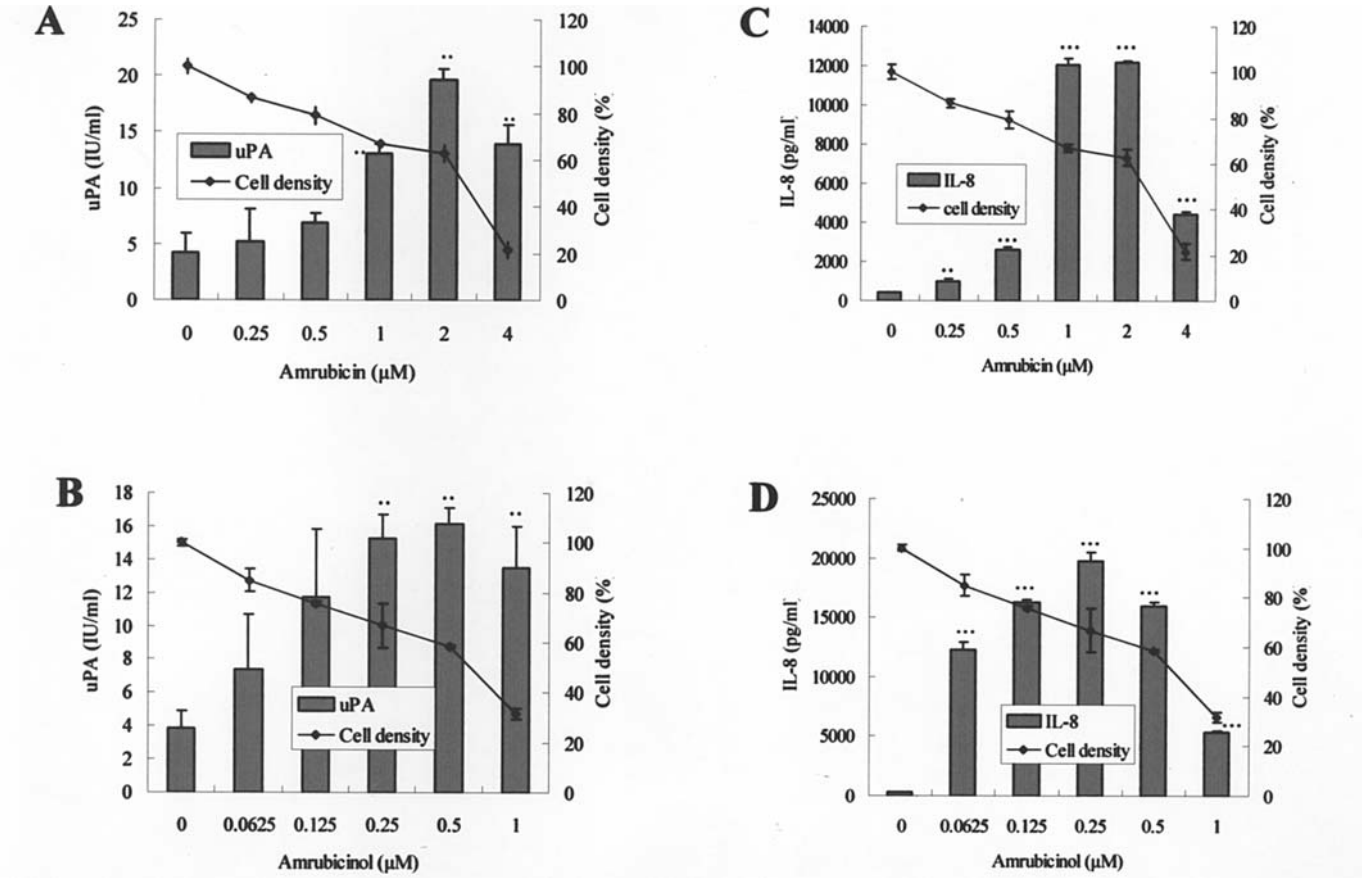

Figure 1. Effects of AMR and AMROH on uPA and IL-8 accumulation and cell density in H69 cells. H69 cells were cultured in the presence of varying concentrations of AMR (A and C) and AMROH (B and D) for 24 h, and uPA activity (A and B), IL-8 levels (C and D) and cell density were measured using a synthetic uPA substrate (S-2444) and MTT assay, respectively, as described in Materials and methods. Each experiment was conducted as least twice in triplicate, and results were reproducible. Columns and closed circles indicate uPA or IL-8 levels and cell density, respectively. Mean ± SE of triplicate values are shown. $^{***}p<0.001$ and $^{**}p<0.01$ using Student's t-test compared to values obtained from AMR- or AMROH-treated cells.

Northern blotting and RNase protection assay. To further clarify the significance of the induction of uPA, IL-8 and MCP-1, we performed an *in vivo* study by subcutaneously injecting the cultured supernatant obtained from AMR or AMROH-treated cells into rabbits. Microscopic examination revealed the migration of a significant number of proinflammatory cells, especially eosinophils, around the injection site. The expression of eotaxins, major eosinophil chemoattrantants, was examined in AMR-treated cells. Here, we demonstrate the AMR-induced eotaxin-3 expression in H69 cells.

Materials and methods

Materials, cell lines and culture. AMR and AMROH were kindly provided by Sumitomo Pharmaceuticals (Osaka, Japan). Human NCI-H69 (ATCC HBT-119) SCLC cells were grown in RPMI-1640 culture medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Whittaker Bioproducts, Walkersville, MD, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were washed once with phosphate-buffered saline and resuspended in serum-free RPMI-1640 (at approximately 1-2x10^6/ml). Cells were exposed to AMR or AMROH for 1 h because AMR and AMROH are usually administered to patients by an intravenous infusion and therefore they rapidly disappear from the blood stream. Cells washed once with serum-free RPMI-1640 were further cultured in serum-free RPMI-1640 in a 24-well culture plate (1 ml/well) or 6-well culture plate (5 ml/well) in a 5% CO_2 incubator. Cell growth was estimated by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertrazolium bromide (MTT) assay for mitochondrial dehydrogenase enzyme activity as described by Mosmann (14), and results were expressed as cell density (% relative to untreated controls). uPA activities in the cultured supernatant obtained 24 h after exposure to AMR or AMROH were measured using a synthetic uPA substrate, S-2444 (15) and a plasminogen-containing fibrin plate (fibrin zymography) as previously described (16). Cultured supernatants were collected and stored at -80˚C before use.

ELISA for IL-8 and eotaxin-3. After stimulating with varying concentrations of AMR or AMROH, the cultured supernatant from each cell type (1.0x10^6/ml) was collected and processed for IL-8 and eotaxin-3 quantification by an ELISA kit (R&D Systems, MN, USA).

Northern blot analysis and RT-PCR. Eotaxin-3 mRNA levels were detected by Northern blotting. Briefly, cells were exposed to AMROH for 1 h, washed once with the RPMI-1640 culture medium, and further cultured for varying times. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method, and 10 μg of total RNA was subjected to Northern blot analysis as described by Sambrook *et al* (17). A

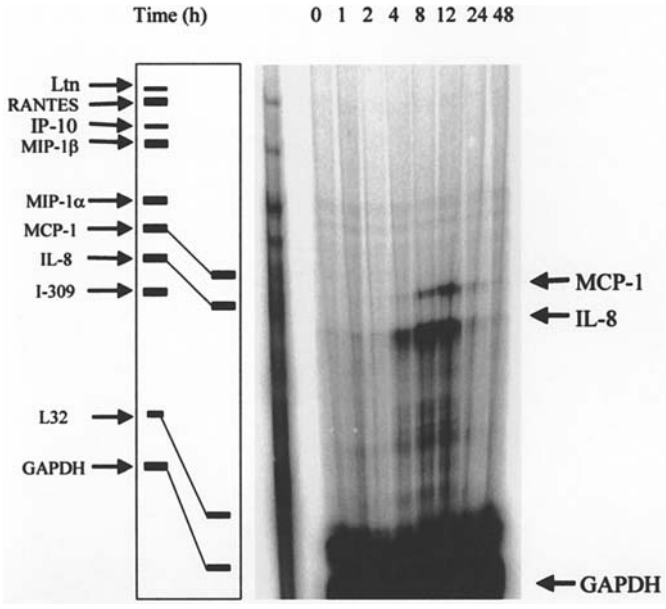

Figure 2. RNase protection assay before and after AMROH stimulation in H69 cells. Total RNA (10 μg) isolated from H69 cells exposed to 0.5 μM AMROH for varying hours was further purified to mRNA using an oligo(dT)-cellulose column and subjected to RNase protection assay, as described in Materials and methods. Lanes on the left illustrate the positions of radio-labeled antisense probe and the remaining 'RNase protected' probes that were resolved on denaturing polyacrylamide gels.

cDNA probe for eotaxin-3 was labeled with 32P-dCTP by a random primed DNA labeling technique. mRNA levels were quantified by counting radioactivity using a BAS-2000 imaging analyzer (Fuji-film, Tokyo, Japan). To control the differences in RNA sample loading, filters were rehybridized with a radiolabeled ß-actin cDNA probe.

RT-PCR was performed as follows: Briefly, total RNA (2 μg) was reverse-transcribed with the SuperScript Preamplication System (Life Technologies, Rockville, MD, USA) using oligo(dT) primers according to the manufacturer's instructions. Aliquots (1 μg) of the reverse-transcribed cDNA were subjected to PCR. The sequences of eotaxin, eotaxin-2, eotaxin-3 and regulated upon activation, normal T-cell expressed and secreted (RANTES) obtained from the studies of Terada *et al* (18), Dulkys *et al* (19), Hoeck *et al* (20) and Tokunaga *et al* (21), respectively, were used to design primers. The sequences of sense and antisense primers for eotaxin were 5'-CCTCTCACGCCAAAGCTCACA-3', and 5'-TAGGCAA CACTCAGGCTCTGG-3', corresponding to nucleotides 63-83 and 437-417, respectively (product size 375 bp). The sequences of sense and antisense primers for eotaxin-2 were 5'-CCATA GTAACCAGCCTTC-3', and 5'-CAGGTTCTTCATGTAC CTC-3', corresponding to nucleotides 17-34 and 267-249, respectively (product size 251 bp). The sequences of sense and antisense primers for eotaxin-3 were 5'-GCCTGATTTG CAGCATCATGATGG-3', and 5'-CGGATGACAATTCA GCTGAGTCAC-3', corresponding to nucleotides 11-34 and 332-309, respectively (product size 322 bp). The sequences of sense and antisense primers for glyceraldehydes-3-phophate dehydrogenase (GAPDH) were 5'-CGGATTTGGTCGTAT TGG-3', and 5'-AGATGGTGATGGGATTTC-3', corres-ponding to nucleotides 87-104 and 289-272, respectively (product size 203 bp). PCR for eotaxin was performed with 30 cycles of a heat-denaturing step at 94˚C for 2 min, a primer annealing step at 55˚C for 2 min and a strand elongation step at 72˚C for 2 min. PCR for eotaxin-2 was performed with 40 cycles of a heat-denaturing step at 95˚C for 30 sec, a primer annealing step at 58˚C for 30 sec and a strand elongation step at 72˚C for 1 min. PCR for eotaxin-3 was performed with 30 cycles of a heat-denaturing step at 94˚C for 1 min, a primer annealing step at 55˚C for 1 min and a strand elongation step at 72˚C for 1 min. PCR for GAPDH was performed with 25 cycles of a heat-denaturing step at 94˚C for 1 min, a primer annealing step at 50˚C for 30 sec and a strand elongation step at 72˚C for 1 min. PCR products (10 μg) were electrophoresed on 2% agarose gel containing 0.5 μg/ml ethidium bromide. Loading was equalized to the internal control mRNA (GAPDH) to give equivalent signals. Gels were illuminated with UV light and photographed using Polaroid film (Polaroid, Herfordshire, UK).

RNase protection assay. Specific detection for mRNA of the chemokine family was performed using the hCK-5 Multi-probe template set (RiboQuant; Pharmingen, San Diego, CA), which contains templates for Ltn, RANTES, IP-10, macrophage inflammatory protein (MIP)-1α, MIP-1ß, MCP-1, IL-8 and I-309, in addition to the housekeeping gene products, L32 and GAPDH. In brief, antisense RNA probes were generated from the DNA templates included in the above assay kit using T7 DNA-dependent RNA polymerase in the presence of [α-^{32}P]UTP (specific activity 3,000 Ci/mmol; ICN, Irvine, CA). Labeled probes were hybridized with total RNA (10 μg) overnight at 56˚C. Unhybridized RNA was digested with RNase according to Pharmingen's instruction manual. RNase-protected probes were resolved on a denaturing 5% poly-acrylamide gel. The gel was dried and visualized using the BAS-2000 imaging analyzer.

Statistical analysis. Statistical analysis was performed on a DOS/V computer (NEC Computer, Tokyo, Japan). Values were expressed as the mean ± SE. Statistical significance was determined using the Student's t-test to compare unpaired data.

Results

Migration of eosinophils around the injection site of the cultured supernatant of AMR-treated cells. We investigated the effects of AMR on uPA, IL-8 and MCP-1 expression in H69 cells. Similarly to the case of doxorubicin, either AMR or AMROH induced uPA and IL-8 in a dose-dependent manner, and the peak induction was observed at a sublethal concentration of each reagent (Fig. 1A-D). Both AMR and AMROH increased uPA accumulation approximately 4-fold, and IL-8, 20-fold, respectively. AMR and AMROH themselves did not directly affect the enzymatic activity of uPA when mixed with standard uPA (Green Cross, Osaka, Japan) in RPMI-1640 medium. The RNase protection assay using human chemokine probes performed as previously described in our study (5) revealed the induction of IL-8 and MCP-1 genes (Fig. 2). To clarify the *in vivo* effects of the cultured supernatant from AMROH-treated cells, we injected subcutaneously the cultured supernatants from AMROH-treated and non-treated cells into rabbits. H69 cells were

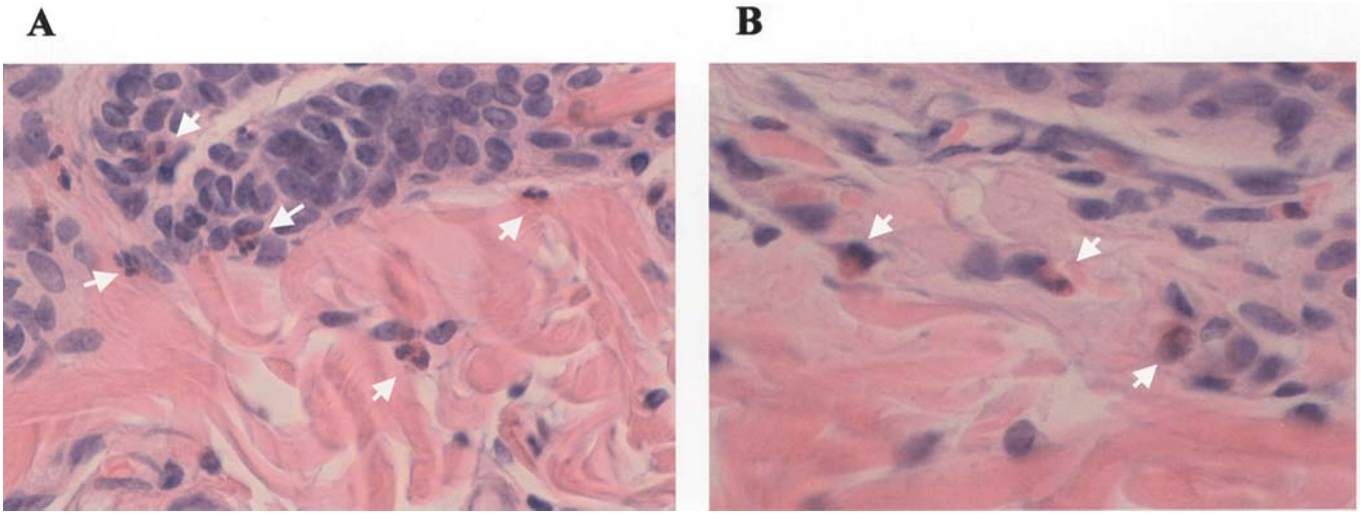

Figure 3. Migration of eosinophils around the injection site of the cultured supernatant from AMROH-treated H69 cells. Specimens taken from the injected site of the cultured supernatant of AMR (A)- or AMROH (B)-treated cells were fixed in formaldehyde and embedded in paraffin. The slides were stained with H&E and visualized by optical microscope. Original magnifications, x400 (A and B). Arrows indicate migrated eosinophils.

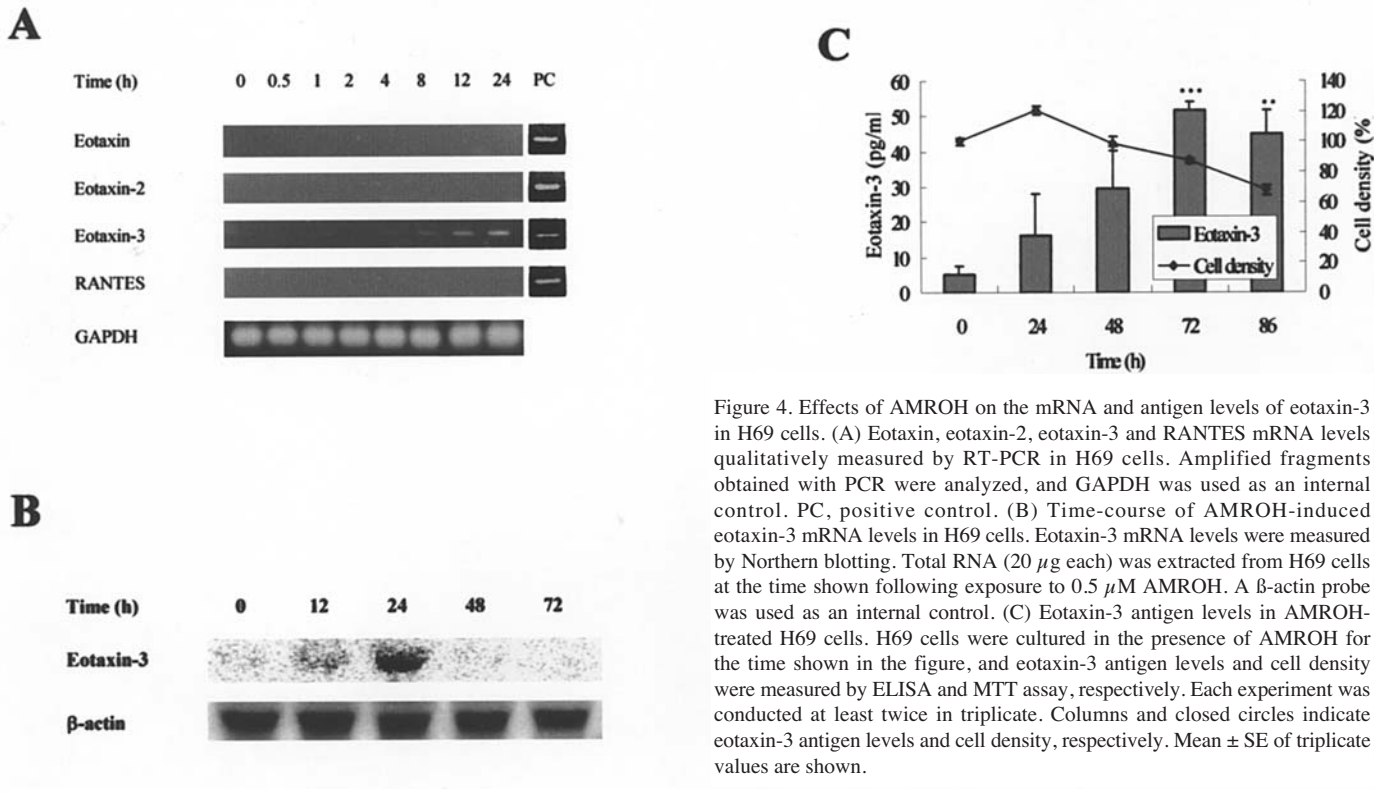

Figure 4. Effects of AMROH on the mRNA and antigen levels of eotaxin-3 in H69 cells. (A) Eotaxin, eotaxin-2, eotaxin-3 and RANTES mRNA levels qualitatively measured by RT-PCR in H69 cells. Amplified fragments obtained with PCR were analyzed, and GAPDH was used as an internal control. PC, positive control. (B) Time-course of AMROH-induced eotaxin-3 mRNA levels in H69 cells. Eotaxin-3 mRNA levels were measured by Northern blotting. Total RNA (20 μg each) was extracted from H69 cells at the time shown following exposure to 0.5 μM AMROH. A ß-actin probe was used as an internal control. (C) Eotaxin-3 antigen levels in AMROH-treated H69 cells. H69 cells were cultured in the presence of AMROH for the time shown in the figure, and eotaxin-3 antigen levels and cell density were measured by ELISA and MTT assay, respectively. Each experiment was conducted at least twice in triplicate. Columns and closed circles indicate eotaxin-3 antigen levels and cell density, respectively. Mean ± SE of triplicate values are shown.

exposed to 0.5 μM AMROH for 1 h, and after washing, further cultured for 23 h in a 5% CO_2 incubator. The cultured supernatant was collected and subcutaneously injected into rabbits and specimens were obtained 24 h after injection by small incision. Microscopic examination revealed the migration of a significant number of eosinophils rather than lymphocytes, neutrophils and monocytes around the injection site (Fig. 3). No significant migration of inflammatory cells including eosinophils was noted around the injection site of the control supernatant.

AMROH-induced eotaxin-3 gene expression. Eotaxin, eotaxin-2, eotaxin-3 and RANTES are known to be migration factors of eosinophils. We investigated their gene expressions in AMROH-treated H69 cells by Northern blotting and RT-PCR. The peak induction of uPA and IL-8 by AMROH was observed at 0.25-0.5 μM (Fig. 1B and D), and, therefore, in this experiment, H69 cells were stimulated with 0.5 μM of AMROH. RT-PCR revealed the induction of eotaxin-3 gene, but not eotaxin, eotaxin-2 or RANTES, in AMROH-treated cells 24 h after stimulation (Fig. 4A). Northern blot analysis

also revealed AMROH-induced eotaxin-3, and the peak was at 24 h after exposure (Fig. 4B).

We also measured the antigen levels of eotaxin-3 in the cultured supernatant by ELISA. AMROH increased eotaxin-3 accumulation in a time-dependent manner as shown in Fig. 4C. This was also dose-dependent (data not shown).

Additionally, we examined the implication of mitogen-activated protein (MAP) kinases in the induction, since we reported the involvement of extracellular signal-regulated kinase (ERK) 1/2 and p38 MAP kinase in doxorubicin-induced uPA expression (22). Treatment with AMROH resulted in the induction of phosphorylation of all three MAP kinases, i.e., ERK 1/2, p38 MAP kinase and c-jun N-terminal protein kinase (JNK) (data not shown). Furthermore, it was suggested by the examination using respective-specific inhibitors against the three MAP kinases that ERK1/2 and p38 MAPK, but not JNK, were implicated in AMROH-induced uPA expression in H69 cells (data not shown). An antioxidant, pyrrolidine dithiocarbamate (1 mM), inhibited the AMROH-induced uPA accumulation (data not shown), suggesting the involvement of ROS in AMROH-induced uPA expression. These results were very similar in the case of doxorubicin (4,22).

Discussion

Eotaxin-3 is a member of the family of CC-chemokines and the most potent chemoattractant for ensionophils. Other important eosinophil chemoattractant cytokines known are IL-5, IL-8, eotaxin, eotaxin-2, RANTES, MCP-3, MCP-4 and TNF-α (23). As shown in this study, it is clear that AMROH induces eotaxin-3 expression as well as IL-8 in a time-dependent fashion in human H69 SCLC cells. Chemoattraction of eosinophils is mainly mediated via the chemokine receptor CCR3. This seven-transmembrane-spanning G protein-coupled receptor is predominantly expressed on eosinophils. CCR3 binds a number of CC-chemokines such as eotaxin and eotaxin-3 (24,25). Thus, eotaxin-3 must have been involved in the migration of eosinophils when the cultured supernatant was injected into rabbit skin in this experiment. CCR3 is also detected on the cell surface of basophils and a subset of Th2 type T lymphocytes (26-28) and, therefore, eotaxin-3 induced by the stimulation with AMROH might chemoattract basophils and Th2 type T lymphocytes, although basophils and Th2 type T lymphocytes were not so significantly migrated in the injection site. Activated eosinophils possess phagocytic capacity, but their main killing mechanism is the release of toxic granule proteins and production of oxygen-free radicals (23). Basophils could release chemical mediators such as IL-4 into the extracellular space of tissues or blood stream and play a significant role in hypersensitivity, hyperplasia, parasitic infections and other diseases (29). Animal models and clinical studies in humans have indicated an important role of Th2 lymphocytes, producing IL-4, IL-5 and IL-13, in the pathogenesis of allergic disorder (30). It is generally suggested that primary exposure to an allergen leads to activation of Th2 lymphocytes in predisposed individuals and stimulation of IgE synthesis of B lymphocytes, and later exposures cause immediate release of chemical mediators and further activation of Th2 cells (31). uPA receptor is expressed on the cell surface of basophils, and uPA appears to be a potent chemoattractant for basophils (32).

A number of cytokines such as the granulocyte-macrophage colony stimulating factor and platelet activating factor have been found to be synthesized and stored in human eosinophils and released from eosinophils. IL-8 could release these cytokines as well as IgA and IgG from eosinophils (33,34). Therefore, IL-8 induced by the stimulation with AMR in tumor cells could release these cytokines from eosinophils migrated around tumor tissue.

IL-8 is a member of the CXC chemokine family and acts as a chemoattractant and an activator of neutrophils (10,11), whereas MCP-1 is one of the CC chemokines and functions mainly as a chemoattractant of monocytes/macrophages (12,13). We anticipated the migration of neutrophils and monocytes/macrophages into the injection site of the cultured supernatant because IL-8 and MCP-1 were accumulated in the cultured supernatant of AMR-treated H69 cells. However, observation of the skin by microscope revealed the migration of eosinophils rather than other leukocytes. Therefore, the induction of eotaxin-3 may be the most relevant phenomenon in the tumor cell biology after exposure to AMR. We may have to examine whether neutralization of eotaxin-3 in the culture supernatants blocks eosinophil accumulation in rat, and whether expression of eotaxin-3 and accumulation of eosinophils induced by AMR occur in other cell lines or in other *in vivo* models. We do not yet have direct evidence indicating the involvement of eotaxin-3 in the eosinophil-chemoattractive effects of AMROH-treated cultured supernatant, but it may be clarified if a neutralizing antibody against eotaxin-3 could be obtained.

We have demonstrated here that AMROH markedly induces not only eotaxin-3 but also uPA, IL-8 and MCP-1 in H69 SCLC cells. Eotaxin-3, uPA, IL-8 and MCP-1 are simultaneously and extensively induced in a certain type of tumor cells by AMROH stimulation and presumably strengthen the anti-tumor effects of AMROH by provoking the interaction between inflammatory/immune cells and tumor cells. Thus, the significance of the AMROH-induced biological active substances would be clarified by future *in vivo* experiments.

Acknowledgements

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