Ultravist induces the expression of MCP-1 and VCAM-1 in IL-4-stimulated HUVECs

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Abstract

The goal of the present study focused on the adverse reaction of contrast medium (CM) via the induction of inflammatory molecules in human umbilical vein endothelial cells (HUVECs). Ultravist-induced MCP-1 and VCAM-1 gene expression was markedly increased in IL-4-pretreated HUVECs in a time- and dose-dependent manner and was paralleled by concomitant production of MCP-1 and VCAM-1 proteins. MCP-1 and VCAM-1 gene expression by Ultravist in combination with IL-4 was mediated by the Jun N-terminal kinases (JNK1/2) signaling pathway. IL-4-pretreated Ultravist-stimulated HUVECs showed greatly increased migration and adhesion of THP-1 cells. Cell migration was decreased by treatment of CCR2 antagonist, and cell adhesion was also decreased by VCAM-1 blocking antibody. Furthermore, when tested in vivo under similar conditions, MCP-1 protein was significantly increased in Ultravist combined with IL-4-injected mice. Taken together, our findings suggest that MCP-1 blocking may be crucial in preventing the endothelial dysfunction induced by contrast medium in patients with inflammatory disease and atherosclerosis.

Keywords: Ultravist, HUVECs, IL-4, MCP-1, cell migration
1. INTRODUCTION

Radiographic iodinated contrast medium (ICM) is the most widely used pharmaceuticals for intravascular administration in diagnostic and interventional angiographic procedures. ICM is relatively well tolerated, and the frequency of use has steadily increased worldwide. However, it has been widely known that symptoms of adverse reactions after ICM injection include nausea, vomiting, headache, itching, skin rash, musculoskeletal pain and fever and that ICM also has toxic effects on renal cells and vascular endothelial cells. One of the leading life-threatening side effects of ICM is acute kidney injury caused by apoptosis and necrosis of renal tubular cells. Most injuries by ICM appear at high doses. However, side effects on endothelial cells by low-dose ICM are not fully understood.

The endothelium is a critical homeostatic structure for the regulation of vascular structure and blood vessel function. Physiologically, the endothelium plays fundamental roles in maintaining a relaxed vascular character by synthesizing and releasing various endothelium-derived vasodilators, such as nitric oxide (NO), prostacyclin (PGI2), endothelium-dependent hyperpolarizing factor (EDHF), and vasoconstrictor endothelin (ET)-1. Endothelial dysfunction is mainly caused by reduced production or action of these relaxing mediators and is the distinct feature of widespread cardiovascular diseases occurring during vasoconstriction, thrombosis, and an inflammatory state.

Interleukin-4 (IL-4) is relatively well known a immunomodulatory cytokine secreted by T helper 2 (TH2) lymphocytes, eosinophils, and mast cells. IL-4 is present at high levels in blood and tissues of patients with chronic inflammatory disease such as asthma, airway inflammation, and atherosclerotic lesions. There is some evidence that IL-4 may play an important role in atheroma formation through the upregulation of monocyte chemoattractant
protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1).\textsuperscript{14,15)}

MCP-1 is a 76-amino acid protein belonging to the CC chemokine family and plays a central role in monocyte chemotaxis and transmigration.\textsuperscript{16)} MCP-1 is expressed and released by a variety of cell types, including vascular endothelial cells, smooth muscle cells, monocytes/macrophages, and fibroblasts stimulated with a number of stimuli, such as inflammatory cytokines, lipopolysaccharide (LPS), platelet-derived growth factor (PDGF).\textsuperscript{17-19)} It is also well known that MCP-1 protein has been detected in the macrophage-rich areas of early atherosclerotic lesions \textsuperscript{20,21)}, and which means that MCP-1 is a potential role in the pathogenesis of atherosclerosis.

The present study was designed to determine the possible mechanisms of human endothelial cell activation resulting from low-dose ICM alone or in combination with cytokines. For this purpose, the effects of various ICMs on the activation of endothelial cells were investigated and the roles of ICM in the IL-4-stimulated endothelial cell activation were subsequently determined.
MATERIALS AND METHODS

Reagents and antibodies

 Ultravist (Berlin, Germany), recombinant human IL-4, IL-6, TNF-alpha and IFN-γ were obtained from Bayer Korea Ltd (Seoul, Korea) and R&D systems (Minneapolis, MN, USA), respectively. The following mAbs were used: anti-phospho AKT, anti-AKT, anti-phospho ERK, anti-ERK, anti-phospho JNK, anti-JNK, anti-phospho p38, anti-p38, anti-phospho STAT1, anti-STAT1 and anti-actin antibodies (Cell signaling and Santa Cruz Biotechnology, Santa Cruz, CA).

Cell line

 The human umbilical vein endothelial cell (HUVEC) was purchased from American Type Culture Collection (Rockville, MD) and maintained in EGM®-BulletKit® (CC-3124, LONZA, Lonza Walkersville, USA) in a humidified incubator at 37 °C and 5% CO2. Human monocytic cells (THP-1) were purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum (Thermo Fisher Scientific hyclone, US), 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Thermo Fisher Scientific hyclone) in a humidified incubator at 37 °C and 5% CO2.

PCR and Real-time PCR

 Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Two micrograms of total RNA was reverse transcribed with SuperScript II reverse transcriptase, and 0.1 µg of the resulting cDNA was PCR amplified using specific primers for MCP-1 (forward, 5’-CAATCAATGCCCCAGTCCT-3’; reverse, 5’-
TCTTCGGAGTTTGGGTTTGCTT-3'), VCAM-1 (forward, 5'- AGC TGC AAG GTT CCT AGC GTGAT -3'; reverse, 5'- ATC TCT GGG GGC AAC ATT GAC  -3') and GAPDH (forward, 5'-CCATGGAGAAGGGCTGGGG-3'; reverse, 5'-CAAAGTTGTCATGGATGACC -3'). The PCR conditions were as follows: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min for 24–30 cycles depending on the amplification product. PCR products were separated using a 1.2% agarose gel and stained with ethidium bromide. For real-time PCR, total RNA was digested with RNase-free DNase (Promega, Madison, WI) to remove contaminating genomic DNA. First-strand cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed using a SYBR Supermix kit and an iCycler system (Bio-Rad, San Diego, CA) according to the manufacturer’s instructions.

EZ-Cytox cell viability assay

Cells were added to 96-well plates at a density of 1 x 104 cells/well and incubated for 6, 12, 24 or 48 hr with various concentration of Ultravist, followed by incubation with 10 μl/well of water soluble tetrazolium salt (EZ-Cytox, Korea) for 1 hr. Cells without any drug were used as a control. Absorbance at 450 nm (A450) was measured with a microplate reader (Model EL800, Bio TEK, USA).

Western blot analysis

Whole cell extracts were prepared in a lysis buffer (5 mM EDTA, 1mM DTT, 1 mM phenylmethylsulfonyl fluoride and 1 x PIC cocktail; sigma). Protein extracts were subjected to SDS–PAGE (10% gel) treatment, transferred to nitrocellulose membranes (Schleicher & Schuell, NH), and probed with each antibodies. The respective bands were detected using HRP-conjugated secondary Ab (Santa Cruz Biotechnology Inc., CA). The amount of
chemiluminescence was measured using a LAS-3000 SYSTEM (Fuji Photo Film, Japan).

**MCP-1 and VCAM-1 expression assay**

The quantities of MCP-1 in the culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). Cells were cultured for 24, 48, or 72 hr in medium containing PBS, Ultravist only, IL-4 only or Ultravist in combination with IL-4 pretreatment for 2 hr, and then the culture supernatants were collected by centrifugation. The levels of MCP-1 were measured using human ELISA MAX Deluxe from BioLegend (SanDiego, CA, USA) according to the manufacturer’s instructions. Data were analyzed with the help of standard curve plotted for this purpose to calculate quantity of MCP-1 (ng/ml). For VCAM-1 expression analysis cultured cells were stained with PE-conjugated anti-VCAM-1 antibody (BD phamingen,USA) and analyzed by flow cytometry.

**Chemotaxis and cell adhesion assay**

Cell migration was performed using a Transwell Permeable Supports (corning, NY). The HUVEC cells were cultured for 72 hr with PBS, Ultravist (20 mg/l/ml), IL-4 (20 ng/ml) or Ultravist in combination with IL-4-pretreatment for 2 hr, in the low chamber of the transwell plate and then cultured additionally for 48 hr. THP-1 cells were stained with calcein-AM for 30 min and then treated with CCR2 antagonist (50 nM; Santa Cruz Biotec) for 30 min. The calcein-AM stained THP-1 cells (5 x 10^5 cells) were added into the upper chamber of the trans-well plate and co-cultured for 1 hr. The number of THP-1 cells in lower chamber was counted. For cell adhesion assay, HUVEC cells (1 x 10^4 cells/well) were cultured in 96 plate for 24 hr and treated with PBS, Ultravist only, IL-4 only, or Ultravist in combination with IL-4-pretreatment for 2 hr. After culture for 72 hr, cells at each well were treated with VCAM-1 blocking Ab (10 μg/ml; BD phamingen, USA) for 1 hr. All cells were washed with PBS and...
incubated with casein AM-stained THP-1 cells (1 x 10^5 cells/well) in fresh culture media for 1 hr. Each well were washed with PBS for two times, and then 100 μl of PBS with 1 % triton X 100 was added each well and read using fluorescence micro-reader.

Mice

Female C57BL/6 mice were purchased from Orient Bio (Seognam, Korea). The animals were kept in isolated cages with a 12-hr light-dark schedule and fed with standard food pellets and water. All of the mice were used at 8–12 wk of age. All of the animal procedures received the approval of the Institutional Animal Care and Use Committee at Inje University College of Medicine.

MCP-1 and \(\beta\)-Hexosaminidase assay

*For assess MCP-1 and \(\beta\)-Hexosaminidase (\(\beta\)-Hex) concentration in serum, mice were injected via a tail vein with PBS, Ultravist (34mg I/mouse) only, IL-4 (200 ng/mouse) only, or Ultravist in combination with IL-4 pre-injection for 2 hr (which means that mice were pre-injected with IL-4 and after 2 hr, followed by re-injection of Ultravist). After 6 hr injection, serum of each mouse was prepared by centrifugation and the quantities of MCP-1 in serum were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. \(\beta\)-Hex, a marker of mast cell degranulation, was quantitated by spectrophotometric analysis according to the manufacturer’s instructions. (Sigma, St. Louis, MO, USA).*

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) of the indicated number of experiments. The statistical significance of data was estimated using a Student’s t-
test for unpaired observations. Multiple comparisons were made using analysis of variance (ANOVA) and the $P$ values less than 0.05 was considered statistically significant.

**RESULTS**

**Cell viability on Ultravist and various cytokines**

Several studies showed that contrast media induce apoptosis of renal cells as well as endothelial cells at high doses.\(^{22,23}\) We first examined the effects of human endothelial cells (HUVECs) by Ultravist. When cells were treated with various doses of Ultravist, cell viability was drastically decreased at doses over 40 mg/l/ml but not lower than 20 mg/l/ml (Fig. 1A). We also measured cell viability by each of the cytokines (Fig. 1B) or Ultravist in combination with various cytokine-pretreated HUVECs (Fig. 1C). Therefore, we decided to use a lower dose of Ultravist (20 mg/l/ml) that did not affect cell proliferation in over 90% of cells. Given that 20 mg/l/ml Ultravist did not affect the viability of HUVECs in pilot studies, this concentration was used in the following experiments.

**Ultravist induces the expression of MCP-1 and VCAM-1 in combination with IL-4**

In the previous study, we found that Ultravist-stimulated human mast cells (HMC-1) expressed various cytokines and chemokines, including IL-4, IL-6, TNF-$\alpha$, and MCP-1.\(^{24}\) Therefore, we analyzed the expression of various cytokines and immune-related genes in Ultravist-stimulated HUVECs. However, Ultravist alone did not induce any cytokines or immune-related genes in HUVECs (data not shown). We next tested the effects of Ultravist on cytokine-pretreated HUVECs. As shown in Fig. 2, low levels of MCP-1 expression were observed in control and Ultravist only-treated cells. However, when IL-4-pretreated HUVECs
were treated with Ultravist, MCP-1 expression was dramatically increased 12-fold compared to control and 2-fold compared to IL-4 only-stimulated cells (Fig. 2A and 2C). Moreover, Ultravist induced VCAM-1 expression 20-fold compared to control and 2.5-fold compared to IL-4 only-stimulated cells (Fig. 2B and 2D). Having demonstrated MCP-1 induction by Ultravist in combination with IL-4, we wanted to determine whether Ultravist-mediated induction of MCP-1 increased production of the MCP-1 protein. We also tested the MCP-1 protein levels in culture supernatants at indicated time points. Consistent with the data on MCP-1 gene expression, treatment with Ultravist resulted in a time-dependent upregulation of MCP-1 protein levels (Fig. 3A). Ultravist also induced VCAM-1 protein levels on IL-4-pretreated HUVECs in a time-dependent manner (Fig. 3B and 3C).

**Ultravist induces the activation of the JNK pathway in IL-4 pretreated HUVECs**

To identify intracellular signaling pathways associated with CM and subsequent gene expression, we measured the phosphorylation of key signaling molecules. We examined potential involvement of the MAPK signaling pathways (i.e., p38, ERK, PI3K, STAT and JNK) in HUVECs. When cells were treated with IL-4 only, STAT-1 was phosphorylated, and Akt and p38 phosphorylation were slightly induced (Fig. 4A). However, when IL-4-pretreated HUVECs were stimulated with Ultravist, JNK and STAT-1 phosphorylation increased (Fig. 4B). Phosphorylation of JNK peaked after 30 min post-Ultravist treatment and was maintained for 120 min. Next, we tested the expression of MCP-1 using various inhibitors of signal transduction pathways. As shown in Fig. 4C, when IL-4-pretreated HUVECs were treated with various inhibitors, AG490 (JAK/STAT inhibitor) completely inhibited the expression of the MCP-1 gene. However, the effects of LY294002 (PI3K inhibitor) and SB203580 (p38 inhibitor) showed mild inhibition. When IL-4 pretreated Ultravist-stimulated HUVECs were treated with various inhibitors of signal transduction
pathways, SP600125 (Jun N-terminal kinase inhibitor) greatly inhibited MCP-1 expression (Fig. 4D). Similarly, VCAM-1 expression in IL-4 pretreated Ultravist-stimulated HUVECs was also inhibited by SP600125 (data not shown). These results confirmed that MCP-1 and VCAM-1 expression in IL-4-pretreated Ultravist-stimulated HUVECs was mediated via the JNK pathway.

**Ultravist inhibits the migration and adhesion of THP-1 cells**

Because MCP-1 expression was greatly increased in IL-4 pretreated Ultravist-stimulated HUVECs (Fig. 2A and 3A), we examined whether Ultravist was involved in immune cell migration. Migration assays were performed using transwells; Ultravist induced mild migration of THP-1 cells similar to the control cells. However, the IL-4-pretreated Ultravist-stimulated HUVECs significantly induced the migration of THP-1 cells by 2-fold compared to IL-4 alone, which resulted in only a slight increase in migration (Fig. 5A). To confirm whether the increased cell migration was decreased by the inhibition of MCP-1 function, the cells were treated with CCR2 antagonist, which is a cell-permeable compound with a high binding affinity for CCR2. As shown in Fig. 5A, when the IL-4-pretreated Ultravist-stimulated HUVECs were treated with CCR2 antagonist, the migration of THP-1 cells was markedly reduced. Furthermore, cell adhesion assays showed that the adhesion of THP-1 cells in culture with the IL-4-pretreated Ultravist-stimulated HUVECs was significantly increased approximately 1.5-fold compared to IL-4-treated cells (Fig. 5B). This level was inhibited by the treatment of VCAM-1 blocking antibody.

**Ultravist induces the expression of MCP-1 and b-Hex in vivo**

To determine whether Ultravist induces the expression of MCP-1 and b-Hex protein in vivo, C57BL/6 mice were injected via the tail vein with PBS (control), Ultravist only, IL-4
only, or Ultravist in combination with IL-4. As shown in Fig. 6A, both IL-4 and Ultravist induced mild MCP-1 levels in mouse serum. Furthermore, MCP-1 levels in IL-4 preinjected Ultravist-injected mice were markedly increased 2-fold compared to the control mice. β-Hexoaminidase levels have been used as an indicator of mast cell activation by Ultravist. In our data, it was also significantly increased in Ultravist in combination with IL-4-preinjected mice compared to control mice (Fig. 6B). Taken together, these results indicated that Ultravist synergistically induces the activation of IL-4-stimulated endothelial cells via JNK activation.
DISCUSSION

The present study investigated whether contrast media (CM) induces adverse reactions in endothelial cells via the release of inflammatory cytokines because exposure to contrast media is connected to adverse cardiovascular events. Several studies have indicated that apoptosis of endothelial cells is induced by high-dose CM; however, the exposure time of high-dose CM was a very short period. Petersein et al.\textsuperscript{24} had found that only 76% of the administered dose (250 mg total iodine/kg body weight) in vivo was eliminated via the urine at 24 hr, whereas concentrations in liver, spleen and blood were still remained 7.7%, 2.5% and 6.3%, respectively. Therefore, we first decided the low-dose CM, which did not affect the viability of endothelial cells, with various nonionic iodinated contrast media, including iobitridol, iohexol, iobrix, and Ultravist. When cells were treated with low-dose contrast medium (20 mgI/ml), the treatment had a minimal effect on cell viability and showed similar results for all of the contrast media used in our pilot studies (data not shown). Therefore, we selected Ultravist for use in the subsequent experiments because Ultravist is a widely used pharmaceutical agent.\textsuperscript{25} It is well known that endothelial cells are directly affected by various cytokines and chemokines in a variety of disease conditions. We also found that Ultravist-stimulated human mast cells (HMC-1) express a lot of cytokines and chemokines, including IL-4, IL-6, TNF-α, and MCP-1 in the previous study.\textsuperscript{25} Thus, we next investigated the effects of cytokines, including TNF-α, IFN-γ, IL-4, and IL-6, but found that only IL-4 affected the function of endothelial cells (data not shown).

CM is regarded as comparatively harmless; however, side effects such as kidney injury have been reported following the intravascular administration of CM. It is the third most common cause of in-hospital acute renal failure (12%), with an incidence of 2-25% after percutaneous coronary interventions.\textsuperscript{26} Furthermore, Seeliger et al. mentioned that renal
failure by CM has been associated with direct cytotoxicity, renal medullary hypoxia, rheological changes, inflammation, and endothelial dysfunction. However, most severe adverse reactions are induced by treatment of high-dose CM, not by low-dose CM.

Hadi et al. showed that the main mediators of endothelial dysfunction in the development of atherosclerosis are vascular inflammatory responses induced by a variety of stimuli. The development of atherosclerotic processes is directly promoted by chemokines. Authors also suggested that critical early pathological events in atherogenesis are caused by the recruitment of inflammatory cells such as monocytes and macrophages and by their migration through the endothelium.

Our studies showed that CM, at a low dose (20 mg/ml), induced the expression of MCP-1 and VCAM-1 in combination with IL-4 (Fig. 2A and 2B). MCP-1 is a chemokine that has a potential role in the pathogenesis of atherosclerosis. Evidence indicates that MCP-1 expression can be induced in response to a variety of proinflammatory stimuli. Nelken and colleagues also found that MCP-1 protein has been detected in early atherosclerotic lesions by immunostaining. Furthermore, Aiello et al. reported that overexpression of MCP-1 accelerated atherosclerosis in apoE-knockout mice. These studies strongly support the idea that the MCP-1-mediated inflammatory environment in the vascular endothelium is critical for the initiation and development of atherosclerosis.

In our data, when HUVECs were treated with Ultravist in combination with IL-4, MCP-1 expression was markedly increased 12-fold compared with control cells (Fig. 2A and 2C). Furthermore, the migration of THP-1 cells was markedly increased in the condition of the IL-4-pretreated Ultravist-stimulated HUVECs (Fig. 5A and 5B). Thus, CM might induce endothelial inflammation as well as atherosclerosis in patients with chronic inflammatory disease, including atherosclerotic lesions because those patients present with high levels of IL-4.
Recent evidence indicates that IL-4 may stimulate the synthesis and secretion of MCP-1 via the transcription factor signal transducers and activators of transcription 1 (STAT1) in human endothelial cells\(^{32}\), but the molecular regulatory mechanism of MCP-1 expression by CM in combination with IL-4 is not yet fully understood. Many studies have used high-dose RCM (not less than 100 mg I/ml), and most cells underwent apoptosis via activation of the p38 or JNK 1/2 pathway.\(^ {22,23}\) In our data, we investigated the molecular signaling pathway of MCP-1 gene expression in IL-4-pretreated Ultravist-stimulated HUVECs. We found that the IL-4-mediated signaling pathway was changed to JNK activation by Ultravist (Fig. 4B). In our results, IL-4 induced MCP-1 expression was completely inhibited in Fig. 4C and 4D when cells were treated with AG490 (Jak-2 inhibitor). Lee et al. also reported that IL-4 induces apoptosis of endothelial cells in combination with cycloheximide (CHX), which inhibits protein synthesis.\(^ {33}\) Based on these results, we speculate that because JAK-2 is located at upper stage to compare with other signaling proteins in signaling pathway, it’s blocking might affect the various effects to proteins in lower stages. Therefore, when treated IL-4 in combination with AG490, it might induce signaling arrest in HUVECs and may completely inhibited MCP-1 expression. On the contrary to this, SP (JNK inhibitor) did not affect IL-4 induced MCP-1 expression (Fig. 4C), but CM induced MCP-1 expression was almost entirely inhibited by the treatment of SP (Fig. 4D). Thus, the transcriptional regulation of MCP-1 expression by IL-4 in human vascular endothelial cells may appear to be synergistically increased by CM.

Taken together, these results indicated that Ultravist synergistically induced MCP-1 and VCAM-1 expression in IL-4-stimulated endothelial cells through JNK activation and that the activation of endothelial cells by Ultravist in combination with IL-4 could enhance the migration and adhesion of immune cells. Therefore, blocking the effect of MCP-1 or VCAM-1 might provide new therapeutic approaches for the control of the contrast media-induced
side effects on endothelial cells.

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Conflict of Interest

The authors declare no conflict of interest.
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Fig. 1. Effects of Ultravist or cytokines in HUVECs. (A) The HUVECs were cultured with PBS or the indicated concentration of Ultravist (0, 10, 20, 40, 80, 100 mg/l/ml) for 48 hr. Cell viability was determined using the EZ-Cytox cell viability assay. Data are presented as the
mean ± SEM of three separate experiments. *P < 0.05 and **P < 0.01, ANOVA. (B) The HUVECs were cultured with various cytokines including IFN-γ (50 ng/ml), TNF-α (10 ng/ml), IL-6 (10 ng/ml), and IL-4 (20 ng/ml) for 48 hr. (C) The HUVECs were pretreated with the indicated cytokines for 2 hr and then cultured with Ultravist (20 mgI/ml) for 48 hr. Cell viability was determined using the EZ-Cytox cell viability assay. Data are presented as the mean ± SEM of three separate experiments.
Fig. 2. Expression of MCP-1 and VCAM-1 in Ultravist-treated HUVECs. The HUVECs were cultured with PBS, IL-4 (20 ng/ml), Ultravist (20 mg/l/ml), or Ultravist in combination with IL-4 pretreatment for 2 hr and then cultured additionally for the indicated times. Total RNA was isolated from these cells and the expression of (A) MCP-1 and (B) VCAM-1 was analyzed by RT-PCR and real-time PCR (C, D). Data are presented as the mean ± SEM of three separate experiments. ***P < 0.001, ANOVA. Ult; Ultravist.
Fig. 3. Ultravist affects the expression levels of MCP-1 and VCAM-1 in IL-4-stimulated HUVECs. The HUVECs were cultured with PBS, Ultravist (20 mgI/ml), IL-4 (20 ng/ml), or
Ultravist in combination with IL-4 pretreatment for 2 hr and then cultured additionally for the indicated times. (A) Culture supernatants were prepared by centrifugation and MCP-1 levels in each sample were analyzed by enzyme-linked immunosorbent assay (ELISA). Data are presented as the mean ± SEM of three separate experiments. ***P < 0.001, ANOVA. (B) Cells were stained with PE-conjugated VCAM-1 antibody and then analyzed by flow cytometry. (C) VCAM-1 expression is presented as the mean fluorescence intensity (MFI) of three separate experiments. ***P < 0.001, ANOVA.
Fig. 4. Analysis of signaling activation and MCP-1 expression in IL-4 stimulated Ultravist-treated HUVECs. The HUVECs were cultured with (A) IL-4 (20 ng/ml) only or (B) Ultravist (20 mg/ml) in combination with IL-4 pretreatment for 30 min and then cultured additionally for the indicated times. Whole-cell lysates were used for western blotting with the indicated antibodies. HUVECs were pretreated with the indicated inhibitors for 1 hr and
then stimulated with (C) IL-4 (20 ng/ml) or (D) Ultravist (20 mgI/ml) in combination with IL-4-pretreatment for 2 hr and then cultured additionally for 24 hr. Total RNAs were prepared and the expression of MCP-1 was analyzed by RT-PCR or real-time PCR. The following treatments were performed: NT (no treatment), AG (STAT inhibitor; 20 μM), LY (PI3K inhibitor; 10 μM), PD (ERK inhibitor; 20 μM), SP (JNK inhibitor; 20 μM), and SB (p38 inhibitor; 20 μM). Data are presented as the mean ± SEM of three separate experiments.
Fig. 5. Ultravist affects the migration and adhesion of THP-1 cells cultured with IL-4-stimulated HUVECs. A) HUVECs (1 x 10^5/12 well) were cultured in the lower transwell chamber for 24 hr. Cells were treated with PBS, Ultravist (20 mg/l/ml) only, IL-4 (20 ng/ml) only and Ultravist in combination with IL-4 pretreatment for 2 hr and then cultured additionally for 48 hr. The THP-1 (human monocytic cell line) cells were stained with calcein-AM for 30 min and then treated with CCR2 antagonist (50 nM) for 30 min. The calcein-AM stained THP-1 cells (5 x 10^5 cells) were added into the upper chamber of the transwell plate and cocultured for 1 hr. The number of THP-1 cells in the lower chamber was counted. Data are presented as the mean ± SEM of three separate experiments. ***P < 0.001, ANOVA.

B) HUVECs (1 x 10^4/96 well) were cultured for 24 hr and treated with PBS, Ultravist only, IL-4 only, or Ultravist in combination with IL-4 pretreatment for 2 hr. After culture for 72 hr, cells in the indicated wells were treated with VCAM-1 blocking Ab (10 μg/ml) for 1 hr. All cells were washed with PBS and incubated with casein AM-stained THP-1 cells (1 x 10^5/96 well) in fresh culture media for 1 hr. Each well was washed with PBS two times, and then 100 μl of PBS with 1% Triton X 100 was added to each well and visualized using a
fluorescence microreader. Data are presented as the mean ± SEM of three separate experiments. ***p < 0.001, ANOVA. Ant; antagonist.
Fig. 6. Ultravist affects MCP-1 and β-hexosaminidase release in IL-4-stimulated mice. C57BL/6 mice were injected via the tail vein with PBS, Ultravist (34 mg I/mouse) only, IL-4 (200 ng/mouse) only, or Ultravist in combination with IL-4-preinjection. After 6 hr of injection, the serum of each mouse was prepared by centrifugation. Levels of MCP-1 and β-hexosaminidase (β-Hex) were quantified by ELISA and the β-hexosaminidase assay. Data are presented as the mean ± SEM (n=4). *P < 0.05 and ***P < 0.001, ANOVA.