Hypoxia-induced IL-18 Increases Hypoxia-inducible Factor-1 α Expression through a Rac1-dependent NF- κ B Pathway

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Submitted February 28, 2007; Revised September 26, 2007; Accepted November 5, 2007
Monitoring Editor: John Cleveland

Interleukin-18 (IL-18) plays pivotal roles in linking inflammatory immune responses and tumor progression and metastasis, yet the manner in which this occurs remains to be sufficiently clarified. Here we report that hypoxia induces the transcription and secretion of IL-18, which subsequently induces the expression of hypoxia-inducible factor-1 α (HIF-1 α). Mechanistically, IL-18 induces HIF-1 α through the activity of the GTPase Rac1, which inducibly associates with the IL-18 receptor β (IL-18R β) subunit, via a PI3K-AKT-NF- κ B-dependent pathway. Importantly, the knockdown of the IL-18R β subunit inhibited IL-18-driven tumor cell metastasis. Collectively, these findings demonstrate a feed-forward pathway in HIF-1 α -mediated tumor progression, in which the induction of IL-18 by hypoxia or inflammatory cells augments the expression of both HIF-1 α and tumor cell metastasis.

INTRODUCTION

A growing body of evidence has been collected suggesting that the activation of hypoxia-inducible factor 1 (HIF-1) renders tumors more aggressive and results in more unfavorable clinical outcomes. HIF-1 is a heterodimeric protein that consists of two subunits, HIF-1 α and HIF-1 β (Harris, 2002). HIF-1 heterodimers bind to the hypoxia response element (HRE), a 5'-RCGTG-3' consensus sequence (Cummins and Taylor, 2005). HIF-1 α is oxygen-labile and is degraded by proteasomes after prolyl-hydroxylation by prolyl hydroxylase and ubiquitination by the von Hippel Lindau (pVHL) complex under normoxic conditions (Hellwig-Burgel *et al.*, 2005). Additionally, the factor inhibiting HIF-1 (FIH) hydroxylates asparagine residues in the C-TAD transactivation domain and inhibits the interaction of HIF-1 α with p300/CBP (Lando *et al.*, 2002).

However, malignant cells evidence a certain degree of HIF activation under normoxic conditions, which implies that nonhypoxic activators can also regulate HIF-1, thereby con-

tributing to the development of cancer. The factors assessed have been utilized to demonstrate that lipopolysaccharide (LPS), interleukin (IL)-1 β , and tumor necrosis factor (TNF)- α can induce HIF-1 α expression under normoxic conditions (Blouin *et al.*, 2004). IL-18, originally referred to as interferon- γ -inducing factor, is a proinflammatory cytokine that belongs to the IL-1 cytokine family (Ghayur *et al.*, 1997; Dinarello, 1999). A wide range of cancer cells can generate and respond to IL-18 via the IL-18 receptor complex (IL-18R α and -18R β). Circulating concentrations of IL-18 were shown to be increased in the presence of metastatic tumors, and a trend has been identified toward worse differentiation of hepatocellular carcinomas that are positive for IL-18 receptor expression, suggesting that IL-18 may participate in the biological progression of aggressive tumor cells (Asakawa *et al.*, 2006).

The process of angiogenesis links many physiological processes to disease states, including tumorigenesis and inflammation (Harris, 2002; Hellwig-Burgel *et al.*, 2005). Certain inflammatory cytokines are known to initiate tumor progress and to maintain angiogenesis (Hellwig-Burgel *et al.*, 2005). IL-18 and -1 share some common signaling pathways and many biologically similar inflammatory functions (Dinarello, 1999). A previous report has shown that the induction of HIF-1 α by IL-1 β performs essential functions in tumor development via the promotion of cell proliferation, invasion, and metastasis (Jung *et al.*, 2003). It has been suggested that IL-18 exerts an antitumor effect via the inhibition of angiogenesis and tumor growth (Nakamura *et al.*, 2006). However, in a recent report, IL-18 has been suggested to function as a novel angiogenic mediator (Park *et al.*, 2001). It has also been previously reported that there was a trend

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-02-0182>) on November 14, 2007.

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Abbreviations used: EMSA, electrophoretic mobility shift assay; HIF-1 α , hypoxia inducible factor-1 α ; IL-18, interleukin-18; IL-18 R β , interleukin-18 receptor β ; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-L-cysteine; PI3K, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

toward worse differentiation in hepatocellular carcinoma patients positive for IL-18 receptor expression, which suggests that certain malignant hepatocellular carcinoma cells acquire the IL-18 receptor during the process of tumorigenesis, and this may contribute to the biological progression of aggressive tumor cells (Asakawa *et al.*, 2006). In addition, IL-18 receptor expression in tumor tissues was shown to be a prognostic marker for poor outcomes (Asakawa *et al.*, 2006). Thus, the role of IL-18 in the regulation of metastasis and angiogenesis in tumors remains controversial.

In our attempts to understand the manner in which IL-18 activates inflammation signals to induce tumor metastatic potentials, we determined that IL-18 was induced under hypoxia and stimulated HIF-1 α production, promoting tumor metastasis via a Rac1-dependent nuclear factor (NF)- κ B pathway.

MATERIALS AND METHODS

Cells and Reagents

The mouse melanoma B16F10 cell lines and human embryonic kidney 293T cells were obtained from the American Type Culture Collection (Manassas, VA). The B16F10 cells were cultured in RPMI medium (Invitrogen, Grandland, NY) and the 293T cells were cultured in DMEM medium in a humidified atmosphere of 5% CO₂. All media were supplemented with 10% fetal bovine serum (FBS), HEPES, and penicillin/streptomycin (Invitrogen). The B16 F10 cells stably transfected with short hairpin RNA (shRNA) against IL-18R β were grown in the presence of puromycin. Recombinant IL-18 was purchased from R&D Systems (Minneapolis, MN). H₂O₂, N-acetyl-L-cysteine (NAC), pyrrolidine dithiocarbamate (PDTTC), and actinomycin D were purchased from Sigma (St. Louis, MO). LY249002, an inhibitor of phosphatidylinositol-3-kinase (PI3K), rapamycin (an mTOR inhibitor), and PD98059, an inhibitor of mitogen-activated protein kinase (MAPK), were all obtained from Calbiochem (San Diego, CA).

Luciferase Assays

For the transient transfection of reporter plasmids, B16 F10 cells (5×10^4) were transfected with plasmids using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's recommendations. After a period of incubation (16–18 h), cells were treated with IL-18 for the indicated times. To assay luciferase activity, the cells were lysed with glo lysis buffer (Promega, Madison, WI) and luciferase activity was determined via standard procedures. All experiments were conducted in triplicate, and luciferase activity was normalized to β -galactosidase activity.

Northern Blot Analysis

Total cellular RNA was isolated from the B16F10 cell lines with Trizol reagent (Invitrogen), in accordance with the manufacturer's instructions. The total RNA samples (20 μ g) were applied to 1.2% agarose/formaldehyde gels and transferred onto nylon membranes. The filters were hybridized with radiolabeled mouse IL-18 and HIF-1 α cDNA probes, washed, and autoradiographed at -70°C .

Western Blot Analysis

The cell lysates were electrophoretically separated via 10% SDS-PAGE and transferred to nitrocellulose membranes. Equal loading and transfer efficiency were confirmed via measurements of protein concentrations as determined by staining with 2% Ponceau S solution. The membranes were blocked at room temperature for 1 h with PBS/5% fat-free skim milk and incubated with monoclonal anti-HIF-1 α antibody (BD Biosciences, Lexington, KY), anti-CXCR4 (Chemicon, Temecula, CA), and anti-vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Santa Cruz, CA). All blots were developed using SuperSignal chemiluminescence substrate (Pierce, Rockford, IL) with anti-mouse horseradish peroxidase IgG antibody (Sigma).

Active Rac1 Assay and Glutathione S-Transferase Pulldown

The B16F10 cells (5×10^6) were stimulated with recombinant IL-18 and lysed with lysis buffer (25 mM HEPES, pH 7.5, 1% Triton X-100, 10 mM MgCl₂, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail) containing 10 μ g of GST-PAK and then incubated for 2 h at 4°C. The cell lysates were cleared via centrifugation and the supernatants were incubated with 30 μ l of glutathione agarose beads for 2 h at 4°C. The bead pellets were washed three times in lysis buffer and finally resuspended in 30 μ l of Laemmli sample buffer. The proteins were separated via 15% SDS-PAGE, and associated active Rac1 was detected via Western blot analysis with an anti-Rac-

specific antibody (Upstate Biotechnology, Lake Placid, NY). For the protein interaction assay, myc-tagged Rac1-expressing plasmid and glutathione S-transferase (GST)-fused IL-18R β -expressing plasmid were cotransfected into 293T cells. After 36 h, the cells were resuspended in lysis buffer. Pulldowns were performed via the addition of 10 μ g of GST-agarose beads to clear the cell lysates. The samples were then incubated for 2 h at 4°C and washed three times in lysis buffer. The precipitated proteins were resuspended in sample treating buffer containing 2% SDS and 5% β -mercaptoethanol. The proteins were analyzed via Western blot analysis with anti-myc antibody (Santa Cruz Biotechnology). For the endogenous interaction, cell lysates were incubated for 2 h at 4°C in the presence of anti-Rac1 antibody, and 15 μ l of protein G-Sepharose beads (50% bead slurry; Roche, Mannheim, Germany) was added to the mixtures. Then, beads were pelleted and washed three times with cold cell lysis buffer (Charrasse *et al.*, 2007). The immunocomplexes were analyzed by Western blotting with anti-IL-18R β antibody.

Electrophoretic Mobility Shift Assay

The nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents in accordance with the manufacturer's instructions (Pierce). In brief, cells (~40 mg) were washed in ice-cold PBS and resuspended in 200 μ l of Cytoplasmic Extraction Reagent 1 (CER1) buffer. The nuclei were collected via centrifugation and resuspended in 100 μ l of NER buffer. After 30 min of incubation at 4°C, the nuclear extracts were collected. The protein concentrations of the extracts were measured via bicinchoninic acid (BCA) assays (Bio-Rad, Hercules, CA). The double-stranded oligonucleotides were synthesized as follows: AP-1, 5'-CTTCTATGTGTCACTTCCTG-3'; NF- κ B, 5'-AGTTGAGGGGACTTCCAGGC-3'; SP1, 5'-ATTCGATCGGGCGGGGGCAG C-3'; IFN Consensus Sequence Binding Protein (ICSBP), 5'-AAGTTGCTTCACTTCTCCC-3'. The oligonucleotides were labeled at the 5' ends with [γ -³²P]ATP and T4 polynucleotide kinase (TaKaRa, Tokyo, Japan). Electrophoretic mobility shift assay (EMSA) was performed as previously described (Kim *et al.*, 2000).

RNA Interference

B16F10 cells were transfected with HIF-1 α small interfering RNA (siRNA)-SMART pool reagent, and GFP siRNA produced by Dharmacon (Lafayette, CO) was utilized as a control (Wesche-Soldato *et al.*, 2005; Hara *et al.*, 2006) with Lipofectamine 2000 (Invitrogen) for 48 h and then exposed in serum-free medium under hypoxic conditions for 6 h. The nucleotide sequences of p65 and Rac1 siRNAs were as follows: p65 (Gene Bank accession no. NM_009045, position 925-943) wild-type siRNA (wt) sense 5'-CUCAAGAUCCGCGGAGUAATT-3' and antisense 5'-UUACUCGGCAGAUUCUAGATT-3'; Rac1 (GenBank accession no. NM_009007, position 415-433) siRNA sense, 5'-GCA-GAC AGACGUGUUCUATT-3' and antisense, 5'-UAAGAACACGUCUGUCUGCTT-3'; p65 and Rac1 siRNAs and control siRNA targeting green fluorescent protein (GFP) were obtained from the Samchully Pharmaceutical Co. (Seoul, Republic of Korea). To rescue p65 siRNA effects, 0.5 μ g of plasmids encoding "wobble" mutants of p65 were transfected with corresponding p65 siRNA into B16F10 cells. The wobble mutant plasmid was derived from pcDNA3-p65 using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Based on the wt p65 siRNA-targeting sequence, a single nucleotide substitution was made at position 582 (C to G) to create the mutant construct. This mutant did not alter the amino acid sequence of the p65, but the nucleotide substitutions rendered that insensitive to siRNA-mediated degradation. The primers for "wobble" mutants construct are 5'-caacactgcgagctgaagatctgcccag-3' and 5'-ctcggcagatcttcagctcgagtggtg-3' were used. Lipofectamine 2000 (Invitrogen) was used to transfect cells with 20 μ M siRNA. The efficacy of these siRNAs transfections in each experiment was ascertained by the pmax GFP plasmid as a positive control. For IL-18R β knockdown, B16F10 cells were treated with short hairpin RNA (shRNA) against the mouse IL-18R β coding region. The target sequence was 5'-CAGACGAACAGCATTG-3' for IL-18R β gene. To construct vector to express shRNA, the oligomers were annealed and ligated into pSUPER-RETRO vector (Oligoengine, Seattle, WA) by using BglII and HindIII. B16F10 cells were transfected with shRNA-expressing vector using Lipofectamine 2000 (Invitrogen) and were selected with puromycin (1 μ g/ml).

RT-PCR and Quantitative Real-Time RT-PCR

Total cellular RNA was extracted with TRIZOL reagent (Invitrogen). One microgram of total RNA was utilized for the reverse transcriptase reaction. cDNA was prepared, and PCR was conducted with mouse IL-18 primers (sense, 5'-GCCTCAAACCTTCAAATCA-3'; antisense, 5'-TGGATCCATTCTCTCAAAGG-3') or HIF-1 α primers (sense, 5'-GAACCTGATGCTTTAACT-3'; antisense, 5'-CAACTGATCGAAGGAACG-3') or AP-1 primers (sense, 5'-AGGCAGAGAGGAAGCGCAT-3'; antisense, 5'-TGGCACCACCTGTTAAGTG-3') or ICSBP primers (sense, 5'-CTGCTCAGGCAGGTGTCAAG-3'; antisense, 5'-AGGCCAGCCATTAGTGGTGAAG-3') or NF- κ B primers (sense, 5'-TTCTGAACCTAAGTTGCGTTGTGCTG-3'; antisense, 5'-CACGGTCTGGGAACCTCTGGAA-3') or bcl-6 primers (sense, 5'-CCCTGTGAAATCTGTGGCACTC-3'; antisense, 5'-ACACGCGGTATTGCACCTTG-3') or VEGF primers (sense, 5'-GGCGTGGTGGTGACATGGT-3'; antisense, 5'-ACCTCACCAAGCCAGCACA-3') or β -actin primers (sense, 5'-AGGC-

CCAGAGCAAGAGAGG-3'; antisense, 5'-TACATGGCTGGGGTGTGAA-3'. Real-time RT-PCRs was conducted using the SYBR Premix Ex Tag (TaKaRa, Tokyo, Japan) with a Thermal Cycler Dice TP800 Real-Time System. The threshold cycle (CT) was determined, and the relative gene expression was calculated as described by Livak and Schmittgen (2001). Real-time RT-PCR analysis of gene expression was conducted using three independent sets of each sample, with β -actin utilized as an endogenous reference. For assessment of spliced and unspliced mRNA levels, total RNA was digested with DNaseI (Sigma, St. Louis, MO) after isolation. Then, Real-time RT-PCR was performed. Primers were designed as follows: Target Real-time PCR Primers: Spliced Primer 1, Sense: 5'-GCCATGTCAGAAGACTCTTGCG-3'; (Exon1-Exon4) Antisense: 5'-TCACAGAGAGGGTCACAGCCA-3'; Spliced Primer 2, Sense: 5'-GGCCGACTTCACTGTACAACC-3'; (Exon3-Exon5) Antisense: 5'-GTCATACAAGGCGCATGTGTG-3'; Unspliced Primer 1, Sense: 5'-GCCATGTCAGAAGACTCTTGCG-3'; (Exon1-Intron1) Antisense: 5'-GCTACTGTCAGATGCCATC-3'; Unspliced Primer 2, Sense: 5'-CGAGCCTTTCATCGCTCCTG-3'; and (Intron4-Exon5) Antisense: 5'-GTCATACAAGGCGCATGTGTG-3'.

ELISA

The cultured supernatants were utilized in order to assay the concentration of mouse IL-18, using ELISA kits (MBL, Nagoya, Japan), and VEGF concentration was determined via the use of ELISA kits obtained from another manufacturer (R&D Systems). These ELISA assays were performed in accordance with the instructions of the respective manufacturers.

Flow Cytometry for CXCR4 Expression

After incubation with IL-18, the B16 F10 cells were washed in PBS and centrifuged for 5 min at 1500 rpm. The cells were resuspended in PBS and transferred to 5-ml culture tubes. To each of the tubes was added the following: 100- μ l cells, 20 μ l of fluorescein isothiocyanate-conjugated anti-CXCR4 mAb (Chemicon) or 100 μ l of isotype antibody (BD PharMingen, San Diego, CA). The mixture was then incubated for 30 min on ice and washed with PBS. The supernatant was removed, and the cells were resuspended in 300 μ l of PBS for flow cytometry.

In Vivo Metastasis Assay

IL-18R β knockdown cell lines and control cell lines were intravenously injected into the lateral tail veins of 6-wk-old C57BL/6 female mice and maintained for 14 d to form tumors in the lung. After 14 d, the mice were killed, and their lungs were removed and washed three times in PBS. The number of nodular metastatic lesions in each lung was counted in order to quantify the metastases.

RESULTS

IL-18 Gene Expression Is Induced under Hypoxic Conditions

To elucidate the functions of IL-18 under hypoxic physiological conditions, we initially attempted to determine whether or not IL-18 expression was induced by hypoxic stimulation. The B16 F10 cells were exposed to hypoxic or normoxic conditions for 6 h. The results of RT-PCR (Figure 1A), Northern blot analysis (Figure 1B), and real-time RT-PCR (Figure 1C) indicated that IL-18 gene expression was dramatically increased under hypoxic conditions. In addition, we also confirmed the elevated secretion of IL-18 under hypoxic conditions (Figure 1D). To further clarify the mechanisms by which hypoxia induces IL-18 expression, the B16 F10 cells were transfected with serially deleted IL-18 P1 promoters and subjected to hypoxic conditions in order to measure the promoter activity. As is shown in Figure 1E, prominent upregulation of promoter activity was observed upon the transfection of the P179 construct. The IL-18 promoter harbors several positive elements, including AP-1, NF- κ B, and ICSBP (Kim *et al.*, 2000), and negative elements including bcl-6 (Takeda *et al.*, 2003). Bcl-6, which is a transcriptional repressor for IL-18 gene expression via the binding of -2574 to -2565 bp of the IL-18 P1 promoter, was also increased under hypoxic conditions. It appears that the full-length IL-18 promoter (p2686) evidences less activity under hypoxic conditions, compared with the truncated promoter (p418), which harbors no bcl-6 binding site as the result of this negative element. Next, the expression of unspliced and

spliced IL-18 transcripts was compared. Real-time RT-PCR results demonstrated that hypoxia increased both forms of IL-18 transcripts (Figure 1F). Overall, these data show that hypoxia induces IL-18 gene expression at the transcriptional level.

Next, in order to determine whether the induction of IL-18 occurred in an HIF-1 α -dependent manner, we utilized HIF-1 α siRNA (Figure 2A). The knockdown of HIF-1 α exerted no effects on hypoxia-induced IL-18 expression, as confirmed via real-time RT-PCR (Figure 2B) and IL-18 ELISA (Figure 2C). These data suggested that hypoxia-induced IL-18 expression occurs independently of HIF-1 α . In addition, no HRE region was detected in the IL-18 P1 promoter, thereby indicating that hypoxia-mediated IL-18 induction is independent of HRE. Previous reports have shown that hypoxia induces the generation of cellular reactive oxygen species (ROS), which appears to participate in the signaling responsible for cytokine secretion (Ali *et al.*, 1999). In an effort to assess the effects of ROS generated during hypoxia on IL-18 induction, IL-18 expression in hypoxic cells was compared with that detected in cells that had been pretreated with the antioxidant, NAC (Supplementary Figure S1). However, we detected no significant reduction of IL-18 expression in the NAC-pretreated cells before hypoxic treatment. These findings indicate that ROS are not involved in the enhanced expression of IL-18 under hypoxic conditions. It has been established that hypoxia induces proinflammatory cytokines, including IL-1 and -6 and TNF- α (Mense *et al.*, 2006). These cytokines may induce IL-18 secretion. When cells were treated with neutralizing antibody against TNF- α , IL-1 β , or IL-6, respectively, hypoxia-induced IL-18 production was not reduced significantly (Supplementary Figure S2, A and B). These results indicate that hypoxia-induced IL-18 production is not mediated by any other proinflammatory cytokines. Collectively, these results demonstrate that hypoxia augments IL-18 gene expression via direct transcriptional activation.

IL-18 Increases HIF-1 α Expression At Transcription Level

Next, in order to determine the effects of secreted IL-18 on HIF-1 α expression, B16F10 melanoma cells were stimulated with recombinant IL-18 for the indicated times. The RT-PCR data showed that the expression of HIF-1 α and VEGF genes were increased significantly in a time-dependent manner as the result of IL-18 treatment (Figure 3A). Northern blot analysis also demonstrated that IL-18 increased HIF-1 α mRNA expression (Figure 3B). In addition, IL-18 increased HRE-promoter activity by about fourfold compared with the control (Figure 3C). To confirm whether or not the effects of IL-18 on the induction of HIF-1 α were transcription-dependent, the cells were treated with recombinant IL-18 in the presence of actinomycin D. In Figure 3D, it was shown that IL-18-induced HIF-1 α expression was inhibited significantly by actinomycin D (top). Meanwhile, actinomycin D exerted only minimal effects on hypoxia-induced HIF-1 α expression (bottom). IL-18 also increased the accumulation of the HIF-1 α protein in a dose-dependent and time-dependent manner (Figures 3, E and F). Under hypoxic conditions, HIF-1 α induction reached maximum levels at 3 h, whereas IL-18-induced HIF-1 α expression increased until the 6-h point. VEGF, one of the target genes of HIF-1 α , has been demonstrated to mediate the metastasis of many types of solid tumors (Ben-Baruch, 2006; Roskoski, 2007). IL-18 also enhances VEGF expression, as verified by ELISA (Figure 3G, left) and Western blotting (Figure 3G, right). Taken together, our results show that IL-18 induces the expression of HIF-1 α as well as its target gene, VEGF.

Figure 1. IL-18 expression is induced under hypoxia. Mouse melanoma cell line B16F10 cells were exposed to hypoxia (1% oxygen) and normoxia (N), after which IL-18 expression was examined at the indicated times. (A) RT-PCR analysis of IL-18, AP-1, PU.1, P300, and CBP. (B) Northern hybridization with IL-18-specific probes on total RNA from hypoxia-stimulated B16F10 cells. (C) The expression of IL-18 mRNAs was tested via real-time RT-PCR in B16F10 cells. The results are expressed as the average of three independent experiments after normalization with the β -actin bands. (D) The level of secreted IL-18 was measured with an IL-18-specific ELISA kit from the culture supernatants of B16 F10 cells incubated under hypoxic conditions for specified times. The data are expressed as the means $\pm$ SD of three independent experiments. (E) Schematic representation of the mouse IL-18 P1 promoter serial deletion constructs and the relative promoter activities on deleted P1 promoter-transfected B16F10 cells under normoxia or hypoxia for the indicated times. The data are expressed as the means $\pm$ SD of four independent experiments. (F) Real-time RT-PCR of spliced and unspliced IL-18 mRNA. Total RNAs under normoxia or hypoxia for 6 h were prepared, and real-time RT-PCR was performed using mouse IL-18 spliced or unspliced primers as described in *Materials and Methods*. The fold changes were normalized to β -actin. The data are expressed as the means $\pm$ SD of three independent experiments.

Figure 2. Hypoxic induction of IL-18 is independent of HIF1- α . (A) HIF-1 α mRNA was knocked down via the incubation of B16F10 cells with 20 μ M HIF-1 α siRNA for 24 h. After IL-18 treatment, total RNA was prepared and the mRNA expression levels of HIF-1 α was evaluated via RT-PCR (top) and Western blotting (bottom). (B) IL-18 mRNA was analyzed via real-time RT-PCR after the incubation of B16F10 cells with 20 μ M HIF-1 α siRNA for 24 h under both normoxia or hypoxia. The results are expressed as the means $\pm$ SD for three independent experiments. (C) IL-18 protein levels were assayed using IL-18 ELISA after HIF1- α siRNA treatment. The results are expressed as the means $\pm$ SD for three independent experiments.

IL-18 Regulates Rac1 Activity to Induce HIF-1 α Expression

We then investigated the signaling molecules responsible for IL-18-induced HIF-1 α expression. Small GTPase Rac1 has been demonstrated to regulate intracellular redox status and to control the activation of HIF-1 α and NF- κ B activation (Wojciak-Stothard *et al.*, 2006), and has also been associated with cell migration and metastasis (Evers *et al.*, 2000). To determine whether Rac1 is required for the functioning of the IL-18-mediated signaling pathway, cells were stimulated with recombinant IL-18 and the cell lysates were applied to the analysis of Rac1 activity, using GST-PAK PBD.

Figure 4A demonstrates that IL-18 induces an increase in PAK-associated Rac1 activity. IL-18R β has been shown to be responsible for IL-18 signaling (Wu *et al.*, 2003). We then attempted to ascertain whether an interaction occurred between Rac1 and IL-18R β . 293T cells were cotransfected with GST-IL-18R β and myc-Rac1, and the cell lysates were immunoprecipitated with GST beads. Rac1 was shown to interact physically with GST-IL-18R β (Figure 4B, top). In addition, the endogenous interaction between Rac1 and IL-18R β was analyzed. It was confirmed that endogenous Rac1 and IL-18R β were interacted each other (Figure 4B, bottom). In an attempt to determine more precisely the functional roles of Rac1 in IL-18-mediated signaling, the effects of a dominant-negative RacN17 mutant were assessed. As a result, IL-18 treatment was shown to induce a sixfold increase in NF- κ B activity, but its stimulatory effects were significantly inhibited by dominant-negative RacN17 (Figure 4C). In addition, Rac1 siRNA reduced IL-18-activated NF- κ B activity, confirming that Rac1 is required for the induction of NF- κ B by IL-18 (Figure 4D). Rac1 regulates intracellular redox status and IL-18 also elicits NF- κ B activity via the regulation of intracellular ROS (Yoon *et al.*, 2004). IL-18- or H₂O₂-induced NF- κ B activity was markedly reduced by treatment with the antioxidant, PDTC (Figure 5A). The combination of RacN17 and PDTC additionally inhibited NF- κ B activity, thereby showing that IL-18-activated Rac1 mediates ROS generation in order to activate NF- κ B. The effects of Rac1 on HIF-1 α expression were subsequently determined (Figure 5B). RacN17 inhibited both NF- κ B activity and HIF-1 α expression, whereas RacV12, an active Rac1 mutant, induced increases in both of them. Diphenylene iodonium (DPI), an inhibitor of NAD(P)H oxidase, also inhibited both NF- κ B activity and HIF-1 α expression. Similar results were observed when RacN17 or RacV12 stable transfectants were established and assessed with regard to NF- κ B activity (Figure 5C) and HIF-1 α expression (Figure 5D). Again, we evaluated the effects of Rac1 siRNA on the induction of HIF-1 α by IL-18. The induction of HIF-1 α by IL-18 was repressed by Rac1 siRNA (Figure 5E). These results show that the association between Rac1 and IL-18R β is involved in IL-18-induced HIF-1 α expression.

IL-18 Increases HIF-1 α Expression through AKT-NF- κ B Pathway

To characterize in greater detail the downstream signaling pathways inherent to IL-18-induced HIF-1 α expression, several inhibitors relevant to HIF-1 α expression were tested. LY-294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor) significantly inhibited IL-18-induced HIF-1 α expression, but PD98059 (MEK inhibitor) evidenced only minimal effects (Figure 6A). Rapamycin inhibited HIF-1 α expression in a dose-dependent manner, coupled with a reduction of mTOR phosphorylation (Figure 6B). LY-294002 also induced a dose-dependent reduction in the expression of HIF-1 α , coupled with a reduction in AKT and mTOR phosphorylation (Figure 6C). In addition, IL-18 itself induced an increase in AKT and mTOR phosphorylation (Figure 6D). Furthermore, when ROS were scavenged by the antioxidants, NAC and PDTC, IL-18 did not up-regulate HIF-1 α (Figure 6E), thereby suggesting that ROS may be essential for the increase in HIF-1 α expression observed in the presence of IL-18. Figure 6F again demonstrated the functions of Rac1 and other signaling molecules including PI3K, AKT, mTOR, and NF- κ B in IL-18-induced NF- κ B activation. In an attempt to determine whether the induction of HIF-1 α by IL-18 is indeed

Figure 3. IL-18 induces HIF-1 α gene expression and target gene. (A) Mouse melanoma B16F10 cells were incubated with recombinant IL-18 under normoxic conditions, and HIF-1 α and VEGF expression levels were determined via RT-PCR. β -actin was used as an internal control. (B) Northern hybridization with HIF-1 α -specific probes of the total RNA from IL-18-stimulated cells for the indicated times. (C) HIF-1 α promoter activity was evaluated with an HRE-luciferase reporter construct. The results are expressed as the means $\pm$ SD of triplicate experiments. All experiments were conducted under normoxic conditions. (D) The cells were pretreated with the transcription inhibitor actinomycin D (10 μ M) and were subsequently treated with either IL-18 (10 ng/ml; top) or exposed to hypoxic conditions (1% oxygen; bottom). After 6 h, the HIF-1 α levels were detected via Western blotting using anti-HIF-1 α antibody. (E) The cells were incubated with recombinant IL-18 for the indicated dosages, and the HIF-1 α levels were detected via Western blotting. (F) The cells were then treated with recombinant IL-18 (10 ng/ml) under normoxic (top) or hypoxic conditions (bottom) for up to 6 h. HIF-1 α levels were determined via Western blotting. (G) The cells were treated with recombinant IL-18 (10 ng/ml) and washed with PBS, and then the levels of VEGF were evaluated via ELISA (left). The error bars represent the means $\pm$ SD from three independent experiments. The cells were treated with recombinant IL-18 (10 ng/ml) time-dependently, and the expression of the VEGF protein was characterized via Western blot analysis (right).

Figure 4. Small GTPase Rac-1 is associated with IL-18 R β physically and functionally. (A) The B16F10 cells were incubated with IL-18 under normoxic conditions for various periods, and coprecipitation using a GST-PBD fusion protein that binds specifically to Rac1-GTP (the active form) was conducted in order to determine the magnitude of Rac1 activation in response to IL-18. (B) 293T cells were cotransfected with a myc-Rac1-expressing plasmid and a GST-IL-18R β -expressing plasmid, and a GST-pulldown assay was conducted using GST beads. The precipitated proteins were then analyzed via Western blotting using anti-myc antibody (top). Cell lysates were immunoprecipitated with anti-Rac1 antibody. Then precipitated proteins were immunoblotted with anti-IL-18R β antibody (bottom). (C) The B16F10 cells were cotransfected with 5 $\times$ NF- κ B reporter plasmid and RacN17-expressing plasmid. After 18 h, the cells were treated with recombinant IL-18 (10 ng/ml) for 6 h and the relative luciferase activity was determined. The values are expressed as the means $\pm$ SD of triplicates. (D) B16F10 cells were infected with Rac1 siRNA or GFPsiRNA. At 24 h after infection, the cells were transfected with reporter plasmids containing 5 $\times$ NF- κ B binding sites for an additional 24 h before stimulation with recombinant IL-18 (10 ng/ml). The cells were harvested at 12 h after recombinant IL-18 treatment, and the luciferase activity was measured. The values are expressed as the means $\pm$ SD of triplicates.

dependent on NF- κ B, we transfected NF- κ B (p65) siRNA into B16F10 cells, and then assessed the levels of HIF-1 α expression. The knockdown of p65 greatly reduced the effects of IL-18 on HIF-1 α expression (Figure 7A). However, the knockdown of HIF-1 α did not affect IL-18-induced NF- κ B activity (Figure 7B). In addition, the effects of enforced expression of the super-repressor mutant of I κ B α (I κ B α -SR) on the induction of HIF-1 α by IL-18 were assessed. I κ B α -SR further suppressed IL-18-induced HIF-1 α expression (Figure 7C). These results demonstrate that IL-18 induces HIF-1 α accumulation via the activation of Rac1-AKT-mTOR and the NF- κ B pathway.

IL-18R β Is the Key Mediator of Oncogenic Signals

The IL-18 receptor is associated with the progression of malignant tumors. IL-18 antisense cDNA and IL-18 binding protein have been previously reported to effect a reduction in tumor metastasis (Carrascal *et al.*, 2003). To determine the prometastatic effects of IL-18 signaling, we utilized siRNA for the IL-18R β retroviral vector in order to down-regulate IL-18R β , which is known to be principally responsible for IL-18 signaling. The IL-18R β knockdown cell lines did not respond to IL-18 (Figure 8A). Thus, we elected to monitor the effects of IL-18R β knockdown on IL-18-induced HIF-1 α

Figure 5. IL-18-activated Rac1 mediates ROS signaling to increase NF-κB and HIF-1α. (A) The B16 F10 cells were cotransfected with 5 × NF-κB reporter plasmids and the RacN17-expressing plasmid. After 18 h, the cells were pretreated with H₂O₂ (100 μM) or PDTC (10 mM) and then treated with or without IL-18 (10 ng/ml) for 6 h, respectively. Relative luciferase activity was measured and the values were expressed as the means ± SD of triplicates. (B) The B16 F10 cells were transfected with RacN17 (N17) or RacV12 (V12). After 18 h, the cells were treated with or without recombinant IL-18 (10 ng/ml) for another 6 h. For DPI treatment, the cells were pretreated with DPI (10 μg/ml) for 1 h. Each of the samples was assayed for NF-κB activity and HIF-1α expression. (C) Establishment of RacN17 and RacV12 cell lines. The B16F10 cells were transfected with RacN17-(N17) or RacV12 (V12)-expressing vectors. After 4 wk, the neomycin-resistant colonies were selected, and the cell clones that expressed RacN17 or RacV12 were identified via Western blotting with anti-myc (top). The identified cell clone was cotransfected with reporter plasmids harboring 5 × NF-κB binding sites. After 18 h, the cells were treated with recombinant IL-18 (10 ng/ml) and the relative luciferase activity was measured after 12 h of IL-18 stimulation (bottom). The values are expressed as the means ± SD of triplicate experiments. (D) The cell clones expressing RacN17 or RacV12 were incubated for 6 h with recombinant IL-18 (10 ng/ml). The level of HIF-1α protein expression was analyzed via Western blotting with HIF-1α-specific antibody. (E) Rac1 mRNA was knocked down via the incubation of B16F10 cells with 20 μM Rac1 siRNA for 24 h. Rac1 expression was measured via RT-PCR (top) and HIF-1α expression by IL-18 was analyzed via Western blotting (bottom).

expression. The induction of HIF-1α expression was suppressed in the IL-18 knockdown cells (Figure 8B). We then assessed the effects of IL-18Rβ knockdown on in vivo tumor metastasis. IL-18Rβ knockdown B16 F10 cells were injected into C57/BL6 mice. After 14 d, the numbers of nodular metastatic lesions were counted in the lungs of each of the subjects. IL-18Rβ knockdown reduced tumor metastasis by more than 80% compared with the controls (Figure 8C). These results demonstrated that the blockage of IL-18 signaling prevents the oncogenic signals involved in in vivo tumor metastasis.

DISCUSSION

The presence of hypoxic regions is a prominent feature of malignant tumors, and tumors occurring in hypoxic regions are associated with poor prognosis and resistance to radiation therapy. Tumor cells also generate cytokines to attract immune cells. In tumor regions, hypoxia can induce enhanced NF-κB activation in alveolar macrophages, which may result in the increased production of inflammatory cytokines. Therefore, the pathogenesis of metastasis relies on multiple interactions between tumor cells and host homeo-

Figure 6. IL-18 induces HIF-1 α expression via PI3K-AKT-NF- κ B pathway. (A) B16 F10 cells were pretreated with LY-294002, PD98059, and rapamycin for 1 h and were incubated in the presence of recombinant IL-18 (10 ng/ml) for 6 h under normoxic conditions, and HIF-1 α levels were determined via Western blotting. (B) Dose-dependent effects of rapamycin on HIF-1 α expression and mTOR phosphorylation were monitored for 6 h in the presence of recombinant IL-18 (10 ng/ml). (C) The dose-dependent effects of LY-294002 on HIF-1 α expression and AKT and mTOR phosphorylation were evaluated in the presence of recombinant IL-18 (10 ng/ml) for 6 h. (D) The effects of IL-18 on AKT and mTOR phosphorylation were monitored for 6 h in the presence of recombinant IL-18 (10 ng/ml). (E) The B16 F10 cells pretreated with NAC (20 mM) or PDTC (100 μ M) were then incubated for 6 h with IL-18 (10 ng/ml). The level of HIF-1 α protein expression was verified via Western blotting. (F) The cells were pretreated with inhibitors or transfected with dnAKT or I κ B α mutant before the addition of IL-18. The cell lysates were analyzed for NF- κ B activity after 6 h of stimulation with IL-18. The values are expressed as the means $\pm$ SD of triplicate experiments.

Figure 7. NF- κ B is required for the induction of HIF-1 α expression by IL-18. (A) The effect of p65 siRNA on p65 expression was confirmed via RT-PCR (top) and the induction of HIF1- α expression by IL-18 was assessed via Western blotting (bottom). HIF1- α levels were abolished by p65 siRNA treatment. (B) B16F10 cells were transfected with HIF1- α siRNA or GFP siRNA. After incubation for 24 h, the cells were transfected with $5 \times$ NF- κ B reporter plasmids. Then, cells were treated with recombinant IL-18 (10 ng/ml) for 6 h, and the relative luciferase activity was determined. The values are expressed as the means $\pm$ SD of triplicates. (C) The super-repressor mutant of the I κ B α (I κ B α -SR) plasmid and control plasmid was transfected into B16F10 cells with or without IL-18 stimulation. HIF-1 α levels were determined via Western blotting. The induction of HIF-1 α by IL-18 was suppressed by I κ B α -SR.

static mechanisms. Recent studies concerning the interaction between tumor promotion and inflammation have shown that inflammatory mediators, including proinflammatory cytokines and nitric oxide, can be abundantly detected within the tumor microenvironment, and that these mediators augment HIF-1 α activity (Hellwig-Burgel *et al.*, 2005). Because the microenvironments of solid tumors are characterized by inflammatory and hypoxic conditions, the regulation of HIF-1 α activity by inflammatory cytokines provides a critical link between cancer progression and inflammation.

In this study, we have demonstrated that IL-18 plays critical roles in the modulation of HIF-1 α expression during tumor progression in several regards; First, IL-18 is induced under hypoxic conditions. Second, secreted IL-18 induces an elevation in HIF-1 α mRNA expression under normoxic conditions. Third, IL-18 activates the Rac1-NF- κ B pathway, thereby altering cellular redox status and signaling, and consequently modulating HIF-1 α expression. These results indicate that IL-18 performs regulatory functions in HIF-1 α expression under both hypoxic and normoxic conditions. As IL-18 is a key inflammatory cytokine and has been shown to be an angiogenic mediator (Park *et al.*, 2001), a close relationship between tumor promotion and inflammation has been proposed, namely that inflammatory mediators serve to augment HIF-1 α activity. In this regard, the production of IL-18 under hypoxic conditions may be another mediator of hypoxia and inflammation. Under these conditions, the induction of HIF-1 α by IL-18 can augment metastatic potential under hypoxic conditions.

Two major pathways are frequently mutated in human cancers. One is Ras—extracellular signal-regulated kinase (ERK); the other is the PI3K—AKT signaling cascades. These are activated by a variety of growth factors, hormones, and extracellular matrix proteins (Pouyssegur *et al.*, 2006). Under hypoxic conditions, Rac1 is known to induce ERK and p38 MAPK activity, resulting in HIF-1 α phosphorylation and an increase in HIF-1 α transcriptional activity (Hirota and Semenza, 2001). Cytokines, including IL-1 β and TNF- α , initiate a cascade of receptor-mediated signaling, which diverges at the level of G-coupled receptors, subsequently activating MAPK or PI3K to up-regulate HIF-1 α biosynthesis. It has been reported that NF- κ B mediates IL-1 β -mediated HIF-1 α expression through COX-2, an NF- κ B-inducible gene (Jung *et al.*, 2003). The IL-1 β -mediated regulation of HIF-1 α stabilization, nuclear translocation, and activation requires a ROS-sensitive mechanism (Haddad, 2002). In addition, biochemical evidence shows that ROS inhibits the activity of HIF (Kheradmand *et al.*, 1998; Werner and Werb, 2002; Chiarugi *et al.*, 2003; Radisky *et al.*, 2005). Meanwhile, our results indicate that the MEK inhibitor, PD98059, inhibited IL-18-induced HIF-1 α expression only partially; however, the PI3 kinase inhibitor LY-294002 induced a significant blockage of IL-18-induced HIF-1 α expression. The p38 MAPK inhibitor SB203580 partially inhibited IL-18-induced HIF-1 α expression at high concentrations. Rapamycin, an mTOR inhibitor, abrogated the IL-18-mediated induction of HIF-1 α .

IL-18 has been determined to be up-regulated after ischemia/reperfusion in the kidney (Daemen *et al.*, 1999), heart (Pomerantz *et al.*, 2001), and proximal tubular cells, as well as in patients suffering from acute coronary syndrome (Mallat *et al.*, 2002). IL-18 is required for the facilitation of neutrophil-dependent injuries via the suppression of anti-inflammatory cytokine expression during hypoxia and ischemia/reperfusion injury (Coban and Aral, 2006). Hepatic IL-18 expression was shown to be up-regulated from 1 h after reperfusion. Redox regulation is also crucial for the control of neovascularization, glucose metabolism, survival, and tumor progression (Pouyssegur and Mehta-Grigoriou, 2006). The Rho family of small GTPases are able to regulate the assembly of the active NAD(P)H oxidase complex, thereby modulating intracellular redox status. Rac1 is activated in response to hypoxia and performs an essential function in the induction of HIF-1 α expression and transcription activity (Hirota and Semenza, 2001). In addition to alterations in cellular redox, both hypoxic and cytokine-induced HIF-1 activation may require the PI3K or MAPK signaling path-

Figure 8. IL-18R β regulates HIF-1 α expression and metastatic potential. (A) Establishment of IL-18R β knockdown cell lines. The B16F10 cells were transfected with the IL-18R β siRNA plasmid. After selection, the cell clones with reduced levels of IL-18R β were verified via RT-PCR. The identified cell clones were then assayed for NF- κ B activity. The values are expressed as the means $\pm$ SD of triplicate experiments. (B) The IL-18R β knockdown cell clone (#2) was incubated for 6 h with IL-18 (10 ng/ml). The level of HIF-1 α protein expression was determined by Western blotting. (C) The IL-18R β knockdown cell clone (#2) was injected into the lateral tail veins of C57BL/6 mice. After 2 wk, the number of nodular metastatic lesions was counted in each lung and is expressed as the average number $\pm$ SD of five independent animals.

ways (Hirota and Semenza, 2001; Jung *et al.*, 2003). Rac1-dependent redox signals perform essential roles not only in cell proliferation and survival (Bedogni *et al.*, 2003), but also in adhesion, detachment and morphological changes, metalloprotease secretion, and epithelial-mesenchymal transition (Kheradmand *et al.*, 1998; Werner and Werb, 2002; Chiarugi *et al.*, 2003; Radisky *et al.*, 2005). In our previous research, we determined that B16F10 melanoma cells generate the IL-18 and IL-18 receptors and that antisense IL-18 reduces ROS levels (Cho *et al.*, 2000). Our present data demonstrate that Rac-1 participates in the IL-18 signaling cascade. The coprecipitation of Rac-1 and IL-18 R β indicate that Rac-1 is a component of the IL-18R β signaling complex. Thus, Rac-1 appears to integrate IL-18R β to facilitate IL-18 downstream signaling for tumor cell metastasis.

In conclusion, the results of this present study show that IL-18-mediated Rac-1 activation and HIF-1 α induction constitute another link between inflammation signals and oncogenic signals. IL-18R β may be a critical connector in this

process and may also provide a molecular basis for the development of novel cancer drugs that target IL-18R β .

ACKNOWLEDGMENTS

This work was supported in part by Grant SC13040 (I.C.) from the 21C Frontier Stem Cell Research Project, Grant GRL from the Ministry of Science and Technology, as well as Grant KRF-2002-015-CS0040 (I.C.) from the Korea Research Foundation, Republic of Korea.

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