Regulation of the Angiogenesis of Acquired Middle Ear Cholesteatomas by Inhibitor of DNA Binding Transcription Factor

Shinji Fukudome, MD; Chuan Wang, MD; Yuki Hamajima, MD; Shengnan Ye, MD, PhD; Yiqing Zheng, MD; Norihiko Narita, MD; Hiroshi Sunaga, MD; Shigeharu Fujieda, MD; Xiaohua Hu, MD; Ling Feng, MD; Jizhen Lin, MD

Importance: The aggressive growth of cholesteatoma in the middle ear involves the angiogenesis of the cholesteatomal perimatrix. However, which transcription factor is involved in this process remains unclear.

Objective: To identify a transcription factor that supports the aggressive growth of cholesteatoma by controlling the angiogenesis of cholesteatoma in the middle ear milieu.

Design: We used clinical specimens for the profiling of angiogenic factors and their regulatory transcription factors in cholesteatoma. Human skin keratinocytes and endothelial cells were used for evaluation of the effects of candidate transcription factor on the angiogenic factor regulation and endothelial cell proliferation.

Setting: University departments of otolaryngology–head and neck surgery.

Participants: Eight clinical cholesteatomal and 8 control specimens were used for cellular and molecular biologic evaluation. An additional 8 cholesteatomal and 8 aural skin specimens were used for microarray studies.

Main Outcome Measures: The expression of vascular endothelial growth factor, interleukin 8, and cyclooxygenase 2 as measured by means of immunohistochemistry and molecular biologic methods.

Results: Human aural cholesteatomal specimens were rich in the expression of angiogenic factors such as vascular endothelial growth factor in the cholesteatomal matrix and perimatrix, accompanied by the transcription factor inhibitor of DNA binding (Id1). We found Id1 to be an essential regulator of vascular endothelial growth factor. In addition, potent angiogenic factors, including interleukin 8 and cyclooxygenase 2, were regulated by Id1 via different molecular mechanisms.

Conclusions and Relevance: The transcription factor Id1 controls the angiogenesis of cholesteatoma through the regulation of vascular endothelial growth factor, interleukin 8, and cyclooxygenase 2, which are responsible for the angiogenesis of cholesteatoma. Id1 may serve as a good target for the treatment of cholesteatomal progression in the middle ear milieu.

However, which transcription factors control the angiogenesis of the cholesteatoma perimatrix in the middle ear milieu remain unclear. Our recent studies suggest that transcription factor inhibitor of DNA binding (Id1) is active in otitis media and the cholesteatoma perimatrix.12,13 The role of Id1 in the activation of endothelial cells14 and the angiogenesis of tumors15,16 prompted us to study whether Id1 plays a role in the angiogenesis of cholesteatoma.

The 3 potent cytokines VEGF, IL-8, and COX-2 are thought to be important angiogenic factors for the growth of new blood vessels in tissue remodeling and tumors.4,5,17 Vascular endothelial growth factor is specific to the proliferation of vascular endothelial cells. Interleukin 8 plays a role in the angiogenesis of many cancer tissues.9,17 Selective inhibition of COX-2 significantly suppresses the angiogenesis in tumor.18 However, whether Id1 regulates the expression of VEGF, IL-8, and COX-2 and controls the angiogenesis of cholesterol remains to be elucidated.

We hypothesized in this study that Id1 regulates the expression of the angiogenic factors VEGF, IL-8, and COX-2 and is thus involved in the angiogenesis of cholesterol. To test this hypothesis, we first determined the promoter activity of VEGF, IL-8, and COX-2 in the presence of Id1 with luciferase assays. The expression of VEGF and IL-8 peptides was then verified with an enzyme-linked immunosorbent assay (ELISA) after transfection of human skin keratinocytes (Rhek-1A) with Id1. To prove the importance of Id1 in the angiogenesis of cholesteatoma, we used immunohistochemistry to examine Id1 expression in cholesteatoma and studied the role of Id1 in the angiogenesis of cholesteatoma in vitro and in vivo.

METHODS

MATERIALS

Eight human aural cholesteatoma tissue specimens and skin tissues were procured from the Fukui Medical University for microarray analysis. Eight normal head and neck skin specimens and cholesteatoma tissue specimens were procured from the University of Minnesota Clinics and Hospitals for immunohistochemistry studies. The diagnosis of these specimens was made clinically and verified pathologically. All tissues were procured, handled, and maintained according to the protocols approved by each institutional review board.

CELL CULTURE

The keratinocyte cell line (nontumorigenic Rhek-1A) was established from the human foreskin and immortalized with SV40,19 maintained in Eagle minimal essential medium (Invitrogen), and supplemented with 10% fetal bovine serum, a combination of penicillin and streptomycin (50 μg/mL), and 1.2% 1-glutamine at a 1:100 dilution (full growth medium). During the transient transfection of cells, reduced serum medium (Opti-MEM; Invitrogen) was used, which was supplemented with 6 μg/mL of transfection reagent (Lipofectamine; Invitrogen) but deprived of serum (transfection medium). We used a cell line derived from human umbilical vein endothelial cells (HUVECs)20 and maintained the cell line in F-12K medium (American Type Culture Collection) containing 0.1-mg/mL heparin, 0.03- to 0.05-mg/mL endothelial cell growth supplement, and 10% fetal bovine serum.20

The Id1 complementary DNA (cDNA) was constructed as previously described.12 Short interfering RNA of Id1 and its control (nonspecific short interfering RNA) were constructed in a similar way, as previously described.21 The pAS5 and pAS0 cDNA constructs were made as previously described.22,23 The COX-2 promoter construct was a gift from Tadashi Tanabe, MD, at the National Cardiovascular Center, Research Institute, Osaka, Japan. The reporter plasmids nuclear factor κB (NF-κB) and β-galactosidase were purchased from Stratagene. The β-galactosidase luciferase activity was used as an internal control for luciferase assays in this study.

IMMUNOHISTOCHEMISTRY

Cholesteatomal tissues from clinical patients were fixed in 10% formalin, embedded in paraffin, cut to a thickness of 4 μm, deparaffinized, and incubated with primary antibodies (Id1 with a 1:400 dilution and NF-κB with a 1:100 dilution [Chemicon International, Inc]) for 90 minutes and biotin-conjugated secondary antibodies (goat anti–rabbit IgG with a 1:2000 dilution [Zymed] and goat anti–mouse IgG with a 1:200 dilution [Vector Laboratories]) using the protocols as previously described.21 Tissue sections incubated with nonspecific antibodies (mouse or rabbit IgG isotype [Zymed]) served as control specimens.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Cells were cultured until approximately 60% to 70% confluence and transfected with Id1 and empty vector for 48 hours in full growth medium. We performed the assay using a VEGF and IL-8 ELISA kit (R&D Systems). Supernatant from cell cultures was added to 96-well plates in which IL-8 and VEGF were attached and incubated for 2 hours at room temperature. After application of the wash buffer (provided in the ELISA kit), plates were incubated with detection antibody conjugated to biotin (1:10,000 dilution [Zymed]) and streptavidin conjugated to horseradish peroxidase (1:2000 dilution [Zymed]) for 2 and 1 hour, respectively. Plates were washed again, and substrate (tetramethylbenzidine [Zymed]) was added to each well. After a 15-minute incubation, 2N hydrogen sulfate was added to each well. Absorbance was read at 450 nm on a microplate ELISA reader (Spectra Classic; Tecan, Inc). The experiment was performed in duplicate.

LUCIFERASE ASSAYS

The Rhek-1A cells cultured in a 12-well plate with 60% confluence were transfected with the Id1 construct at 1.4 μg/mL for Id1 protein expression; cotransfected with IL-8, VEGF, and COX-2 luciferase reporters at 1.4 μg/mL for 8 hours in the transfection medium; and incubated in full growth medium for a 24-hour recovery. The β-galactosidase reporter was used as an internal control for transfection efficiency. To study whether regulation of VEGF, IL-8, and COX-2 by Id1 was dependent on the signaling pathway for MEK1/2, IκBα/β, and NF-κB, Rhek-1A cells were cotransfected with IκBα M (1.4 μg/mL, a specific dominant negative inhibitor of NF-κB) for 16 hours, recovered in full growth medium for 24 hours, incubated with U0126 (10 μM, a specific MEK1/2 inhibitor) for 8 hours, and then harvested for luciferase assays. The activities of luciferase reporters are presented as a ratio of luciferase to β-galactosidase reporter activity (ie, relative luciferase activity).
Figure 1. Cholesteatomas are rich in the expression of angiogenic factors and relevant transcription factors. A, Microarray data demonstrated that angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGFA) were upregulated in the cholesteatomal tissues in comparison with the aural skin tissues (n=8). MAPK indicates mitogen-activated protein kinase p38. B, Compared with control specimens (left), the middle ear cholesteatomal perimatrix was rich in blood vessels, and immunohistochemical staining in these blood vessels was positive for inhibitor of DNA binding (Id1) (arrows [middle]) and nuclear factor κB (NF-κB) (arrows [right]). C, In the cholesteatoma perimatrix and matrix, immunohistochemical staining was positive for Id1 in the endothelial cells (arrows [middle]) or on the edge of the cholesteatomal matrix (arrows [right]). The bars in B and C, each applying to the entire row of images, indicate 50 µm.

Figure 2. Inhibitor of DNA binding (Id1) regulates the promoter activity of vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), and cyclooxygenase 2 (COX-2) in Rhek-1A cells. Bars indicate mean; limit lines, standard deviation. A, Luciferase assays demonstrate that Id1 significantly increased the promoter activity of VEGF, IL-8, and COX-2 at 8 hours compared with controls in Rhek-1A cells (n=6). *P<.05, Id1 vs empty vector. B, The increased promoter activity of IL-8 induced by Id1 was abrogated by Id1κBα mutant (n=6). *P<.05. C, Similarly, the increased promoter activity of VEGF induced by Id1, 1.4 µg/mL, was abrogated by U0126 (n=6). *P<.05. RLA indicates relative luciferase activity, presented as the ratio of luciferase to β-galactosidase reporter activity; Med, medium, which served as a control of U0126. D, As determined with the use of an enzyme-linked immunosorbent assay, Id1 significantly increased the protein levels of VEGF and IL-8 in Rhek-1A cells compared with their controls (n=4). *P<.05.
Inhibitor of DNA binding (Id1) increases the intake of thymidine and number of human umbilical vein endothelial cells (HUVECs). Endothelial cells were transfected with Id1 and empty vector (n=3). Data points indicate mean; limit lines, standard deviation. A, Id1 transfection in HUVECs increased the thymidine incorporation in a time-dependent manner. On days 2 and 3, Id1 significantly increased the thymidine intake compared with the empty vector. CPM indicates counts per minute. B, By Trypan blue exclusion, Id1 significantly increased the number of HUVECs compared with the empty vector. HPF indicates high-power field. *P<.05 compared with the empty vector.

**Figure 3.** Inhibitor of DNA binding (Id1) increases the intake of thymidine and number of human umbilical vein endothelial cells (HUVECs). Endothelial cells were transfected with Id1 and empty vector (n=3). Data points indicate mean; limit lines, standard deviation. A, Id1 transfection in HUVECs increased the thymidine incorporation in a time-dependent manner. On days 2 and 3, Id1 significantly increased the thymidine intake compared with the empty vector. CPM indicates counts per minute. B, By Trypan blue exclusion, Id1 significantly increased the number of HUVECs compared with the empty vector. HPF indicates high-power field. *P<.05 compared with the empty vector.

**MICROARRAY ANALYSIS**

Microarray analysis on human aural cholesteatomal matrixes was performed as previously described. Changes in genes of interest (involvement in angiogenesis), such as VEGF (NCBI EntrezGene M32977) and platelet-derived growth factor (PDGF; GenBank X06374), were calculated by normalization of data with the housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]), and final results of genes of interest are presented in fold changes.

**STATISTICAL ANALYSIS**

We used the t test to evaluate differences between the control and experiment factors in vitro or correlates between microvesSEL counts and angiogenic factors whenever appropriate. P < .05 was considered significant.

**RESULTS**

**EXPRESSION OF ANGIGENIC FACTORS IN THE MIDDLE EAR CHOLESTEATOMAL PERIMATRIX**

To study whether cholesteatoma is active in angiogenesis, microarray analysis was performed on 8 clinical cholesteatomal specimens as described in the “Methods” section. We found that messenger RNA transcripts relevant to angiogenesis (VEGF, platelet-derived growth factor A, and matrix metalloproteinases) were upregulated in the middle ear cholesteatomal specimens compared with the 8 skin tissue samples (Figure 1A). To investigate whether angiogenesis is active in the middle ear cholesteatomal perimatrix and whether Id1 and NF-κB are involved in the regulation of these angiogenic factors, immunohistochemistry was performed on additional clinical cholesteatomal specimens. Abundant blood vessels were present in the middle ear cholesteatomal perimatrix, in which Id1 and NF-κB were actively expressed in the endothelial cells (Figure 1B). In the middle ear cholesteatomal matrix, findings for Id1 were highly positive in the blood vessels of the perimatrix and in the cholesteatomal matrix (Figure 1C) and positive in the cholesteatomal perimatrix and matrix. In the cholesteatomal matrix, findings for Id1 were highly positive on the edge of the cholesteatomal matrix in which keratinocytes are proliferative and in the perimatrix in which endothelial cells are active in proliferation.

**EXPRESSION OF VEGF, IL-8, AND COX-2 IN CULTURED SKIN KERATINOCYTES**

To study whether Id1 regulates VEGF and IL-8 at the promoter level, luciferase assays were performed on skin keratinocytes. As expected, Id1 significantly increased the promoter activity of VEGF, IL-8, and COX-2 compared with empty vector controls in Rhek-1A cells (Figure 2A). In addition, the promoter of COX-2 and IL-8 contains a NF-κB binding site in some cells. To study whether a NF-κB binding site is also contained in keratinocytes, Rhek-1A cells were transfected with Id1 and incubated with and without U0126 or IkBo mutant and harvested for evaluation of IL-8 and VEGF promoter activity. We found that Id1-induced promoter activity of IL-8 was blocked by IkBo mutant (Figure 2B), whereas Id1-induced promoter activity of VEGF was abolished only by U0126 (Figure 2C). The ELISA findings demonstrated that Id1 significantly increased the protein expression of IL-8 and VEGF (Figure 2D).

**PROLIFERATION OF HUVECs**

To verify the importance of Id1 in the proliferation of endothelial cells in the cholesteatomal perimatrix, HUVECs were transfected with and without Id1. We found that Id1 significantly increased the uptake of thymidine in HUVECs in a time-dependent manner (Figure 3A). To investigate whether Id1 increases the proliferation of human endothelial cells, cell counting was performed in HUVECs transfected with Id1 and empty vector. We found that Id1 transfection in HUVECs increased the cell number of HUVECs in vitro (Figure 3B).

**COMMENT**

As shown in this study, the angiogenesis in the cholesteatomal perimatrix is attributed to the inflammation-related transcription factor Id1 that is involved in the ag-
The transcription factor Id1 plays a critical role in the angiogenesis of cholesteatoma based on the in vivo and in vitro data. First, Id1 is upregulated in the middle ear cholesteatoma specimens, as shown in recent studies. Second, Id1 is able to increase the expression of VEGF, IL-8, and COX-2 in keratinocytes in vitro. Third, Id1 directly increases the proliferation of human endothelial cells. Together these data strongly suggest that the Id family is a potential target for antiangiogenic therapy in cholesteatoma.

Submitted for Publication: August 17, 2012; final revision received September 30, 2012; accepted November 6, 2012.

Correspondence: Jizhen Lin, MD, Department of Otolaryngology–Head and Neck Surgery, University of Minnesota, 2001 Sixth St SE, Minneapolis, MN 55455 (linxx004@umn.edu).


Conflict of Interest Disclosures: None reported.

Funding/Support: This study was supported in part by grant R01008165 from the National Institutes of Health, R01 supplement 00010055, the National Organization for Hearing Research, and 5M Lions International Foundation.

REFERENCES


