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The role of inhibitor of DNA-binding (Id1) in hyperproliferation of keratinocytes: the pathological basis for middle ear cholesteatoma from chronic otitis media

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Abstract

Objectives: A hallmark of cholesteatoma is hyperproliferation of keratinocytes with abundant production of keratins in the middle ear under chronic inflammatory conditions. However, little is known about the driving force of cellular proliferation and keratin production of cholesteatomal matrix. The purpose of this study was to investigate the cellular proliferation and keratin production of keratinocytes under the influence of Id1, a candidate transcription factor to cell proliferation.

Materials and methods: Keratinocytes were transfected with Id1 and the responses of keratinocytes to Id1 were studied by using cellular and molecular biologic methods.

Results: Id1 positively contributed to the cell cycle progression and negatively to the $p16^{Ink4a}$ downregulation *via* the nuclear factor-kappa B (NF-κB)/cyclin D1 pathway. Id1 significantly increased the promoter activity of NF-κB which, in turn, up-regulated the expression of cyclin D1 and keratin 10 in keratinocytes. Specific NF-κB inhibitors (pyrrolidine dithiocarbamate, PDTC), or dominant-negative inhibitor (I kappa B alpha mutant, IκBαM) abrogated the Id1-induced cell proliferation and keratin 10 production whereas p65, a subunit of the NF-κB heterodimer and an enhancer of the NF-κB activity, strengthened the Id1-induced cell proliferation and keratin 10 production.

Conclusions: Id1 contributed to hyperproliferation of keratinocytes *via* enhancement of cell cycle progression, removal of cell cycle inhibition, and simultaneously increased keratin production.

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Introduction

Chronic otitis media (COM) is linked to cholesteatoma, a condition of aggressive proliferation of keratinocytes and abundant production of keratins, in the middle ear. Morphologically, cholesteatoma forms an epithelial cyst that grows aggressively and erodes the ossicular chain and bony wall of the middle ear. It represents a threat to neighbouring tissues or organs; however, little is known about the driving force behind this abnormal growth of the epithelial cyst and abundant production of keratins in cholesteatoma. Recently, we found that expression of Id1 was up-regulated in the middle ear mucosa of rats following bacterial infection (1) and in the middle ear mucosa of humans with COM and/or cholesteatoma (2). It suggests that Id1 plays a role in pathogenesis of middle ear cholesteatoma.

The *Id* gene family encodes four related proteins, from Id1 to Id4, which are involved in control of cell cycle progression in a wide range of organisms, from flies to humans (3,4). Id proteins are involved in cell population growth and proliferation (3,5,6) by antagonizing actions of basic helix–loop–helix (bHLH) transcription factors, which are essential for cell differentiation. According to the literature, forced expression of Id1 in primary human keratinocytes leads to cell lifespan extension and cell immortalization (7,8). Transfection of the rat middle ear with Id1 *in vivo* has resulted in proliferative responses in the mucosal layer (9). These led us to examine the role of Id1 in proliferation of keratinocytes and production of keratins.

In this study, we hypothesized that Id1 induced proliferation of keratinocytes through up-regulation of NF-κB/cyclin D1, a signalling pathway leading to G_0- to

S-phase progression, and down-regulation of $p16^{Ink4a}$, an inhibitor for suppression of cyclin-dependent kinase (cdk) activity. In addition, we hypothesized that Id1 induced production of keratin 10, one of the major keratin products of keratinocytes, *via* NF-κB. To test these hypotheses, cell and molecular biological experiments were performed (i) on human middle ear cholesteatoma specimens, to evaluate importance of Id1-induced signalling in pathogenesis of cholesteatoma and (ii) on cultured human keratinocytes to verify the signalling pathway.

Materials and methods

Fourteen cholesteatoma tissue specimens were procured from Ichinomiya City Hospital in Nagoya, Japan, Cincinnati Children's Hospital Medical Center (CCHMC) and University of Minnesota Hospitals and Clinics (UMHC). Eight normal (no history of otitis media) middle ear mucosal; head and neck skin specimens procured from UMHC served as controls. Diagnosis of these specimens was made clinically and verified pathologically. All tissues were procured, handled and maintained according to protocols approved by each Institutional Review Board (IRB).

Rhek-1A cell line, representative epidermal keratinocytes (10), was maintained in Eagle's minimal essential medium (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS), penicillin/streptomycin (50 μg/ml) and 2 mM L-glutamine (herein referred to as full growth medium, FGM). During transient transfection of cells, Opti-MEM supplemented with 6 μg/ml of Polybrene® was used (Invitrogen, herein referred to as transfection medium). Rhek-1A was chosen because it is derived from human skin keratinocytes as is cholesteatoma.

Full-length Id1 cDNA was cloned into a protein-expressing vector (pEGFP; Clontech, Mountain View, CA) using standard protocols as described previously (11); functionality of this Id1 construct has recently been verified (12). I kappa B alpha mutant (IκBαM, a kind gift from Dr Inder Verma of the Salk Institute, La Jolla, CA), in which serine at position 36 had been changed to alanine, thus preventing phosphorylation and subsequent proteasomal degradation in response to stimuli (13,14), is a dominant-negative inhibitor of NF-κB activity. Pyrrolidine dithiocarbamate (PDTC), a proteasome inhibitor purchased from Calbiochem, was used as a potent inhibitor of NF-κB (15) and proven as an effective inhibitor of the NF-κB activity in keratinocytes (16). Cyclin D1 reporters were generous gifts of Dr Richard Pestell (Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY). They contain wild-type and mutant cyclin D1 reporter constructs, as described previously (17). Briefly, wild-type cyclin D1 reporter construct consisted of a fragment of cyclin D1 promoter sequence (from −1745 to 0) flanking the *cyclin D1* gene. Mutant cyclin D1 (CDmt) reporter construct has a fragment of cyclin D1 promoter sequence (from −66 to 0) with NF-κB binding site truncated. NF-κB reporter, provided by Dr Keith Brown at the National Institute of Allergy and Infectious Diseases, contains three repeats of κB sites for immunoglobulin κ-light chain (18). β-galactosidase (β-gal) reporter was purchased from Stratagene and used as internal control for NF-κB and cyclin D1 reporters. Id1 signalling pathway and relationship among IκBαM, NF-κB (p65 and p50 heterodimer), PDTC, cyclin D1 and their effectors are schematically presented in Fig. 1.

Immunohistochemistry

Cholesteatoma and control specimens were fixed in 10% formalin, embedded in paraffin wax and cut to a thickness of 5 μm. Sections were deparaffinized, and incubated with primary antibodies to Id1 (rabbit anti-human Id1; Santa

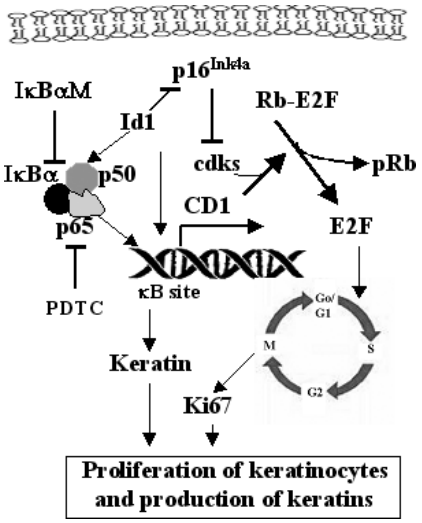

Figure 1. Schematic representation of Id1-induced cell proliferation and keratin 10 production pathways in keratinocytes. Id1 activates translocation of NF-κB subunit p65 into the nucleus and increases transcription of cyclin D1 (CD1). CD1, together with cyclin-dependent kinases (cdk 4/6), phosphorylates retinoblastoma (Rb) protein that releases E2F from the Rb-E2F complex. E2F, a transcription factor that drives the G_0 to S phase transition of cells, promotes cell cycle progression. Ki67 is a proliferating cell antigen active from G_1 to M phase; with expression of Ki67, a cell proliferates. In addition, phosphorylation of NF-κB subunit p65 and transcription of p65 by Id1 increases production of keratins. Id1-induced down-regulation of $p16^{Ink4a}$, an inhibitor of cdks, also promotes cell cycle progression. IκBαM inhibits NF-κB activity by forming a firm complex with p65 and p50, whereas PDTC inhibits NF-κB activity by suppressing formation and degradation of IκB (an inhibitor of NF-κB). NF-κB, consists of a heterodimer: p65 and p50; T-bar, indicating inhibition; arrow, symbolizing up-regulation.

Cruz Biotechnology Inc, Santa Cruz, CA, USA), keratin 10 (rabbit anti-human K10; NeoMarker, Fremont, CA, USA; mouse anti-human pan-cytokeratin, Abcam, Cambridge, MA, USA), 'activated' NF-κB (anti-NF-κB subunit p65; Chemicon, Temecula, CA, USA), cyclin D1 (BD Sciences, Franklin lakes, NJ, USA) and Ki67 (Abcam), for 90 min followed by secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC), using protocols described previously (19). Tissue sections incubated with non-specific antibodies (mouse or rabbit IgG isotope controls from Zymed) or antibody absorbed with specific antigen (Id1 blocking peptide, Santa Cruz Biotechnology Inc, 5-fold excess) at 4 °C overnight, or at room temperature for 2 h, served as controls.

Incorporation of [^{3}H]dT and trypan blue exclusion

Cells were cultured in 24-well plates until 40% confluent, transfected with Id1 and empty vector, respectively, at concentration of 1.4 μg/ml for 16 h in transfection medium, recovered in FGM for 24 h after transfection, incubated with [^{3}H]dT (1 μCi/well) for 5, 10 and 24 h respectively, to measure radioactivity in a scintillation counter, as previously described (20). Simultaneously, cells in duplicate 24-well plates were harvested for cell counts using the trypan blue exclusion technique. Radioactivity per well was divided by cell number in a duplicated well yielding radioactivity on average per cell. Results are presented as CPM/10^4 cells for [^{3}H]dT incorporation. Cell population growth rate (0 versus 5, 10 and 24 h respectively) was calculated using total cell numbers at 0 h against 5, 10 and 24 h respectively.

Luciferase assays

Cells were cultured in six-well plates until 60% confluent, transfected with empty vector, Id1, p65 and Id1+p65, co-transfected with NF-κB/β-galactosidase reporters and cyclin D1/β-galactosidase reporters, respectively, at 1.4 μg/ml for 7–16 h, recovered in FGM for 24 h, and harvested for luciferase assay, as previously described (21). Activities of target luciferase reporters versus β-galactosidase are presented as relative luciferase activity (RLA).

Western blot analysis

Cells were cultured in T-75 flasks up to 60% confluence, transfected with empty vector, Id1, p65, IκBαM, Id1+p65, Id1+ IκBαM and Id1+PDTC, respectively, in transfection medium, for 16 h, recovered in FGM for 24 h, and harvested for cytosol and nuclear protein isolation. Forty micrograms of cytosol or nuclear protein was electrophoresed and blotted on a nitrocellulose membrane. Specific antigens (cyclin D1, keratin 10, p16^{Ink4a} and GAPDH) on the membrane were detected by respective antibodies (anti-cyclin D1, BD Biosciences product; anti-K10 and anti-p16^{Ink4a}, BioMarkers product; anti-GAPDH, Novus Biologicals, Littleton, CO) using the ECL kit (Amersham Biosciences) according to the manufacturers' instructions.

Statistical analysis

Student's *t*-test for unequal variances was used for evaluation of two-group studies, whereas analysis of variance (ANOVA) was used for evaluation of multiple-group studies. P values <0.05 were considered significant.

Results

Id1, NF-κB, cyclin D1, Ki67 and keratin 10 were highly expressed in human middle ear cholesteatoma specimens

By immunohistochemistry, Id1, NF-κB, cyclin D1, Ki67 and keratin 10 were detected in the epithelial layer of cholesteatoma specimens (11 of 14 specimens). Three of 14 middle ear specimens contained onion peel-like substances but no epithelial layer. Normal middle ear mucosa showed no staining for Id1 and activated NF-κB in the epithelial layer (Fig. 2a,b), whereas normal head and neck skin showed baseline expression of Id1 and NF-κB in the basal cell layer (limited to few basal cells, data not shown). Unlike normal head and neck skin specimens, Id1 and NF-κB were extensively expressed in the cholesteatoma specimens. Id1 and NF-κB, NF-κB and cyclin D1, and cyclin D1 and Ki67 antigens were co-expressed in the basal cells (Fig. 2d–h, nuclei) of cholesteatoma, whereas NF-κB and keratin 10 were co-expressed in the suprabasal cell layer (Fig. 2i, nuclei and cytosol) of the cholesteatomatous epithelium.

Id1 induces proliferation of keratinocytes via NF-κB in vitro

To study the role of Id1 in cholesteatomatous epithelium, Id1 was transfected into Rhek-1A cells and cell proliferation was studied by [^{3}H]dT incorporation and cell population growth rate methods. Id1 significantly increased DNA synthesis (Fig. 3A) and cell population growth rate (Fig. 3B), associated with increase of NF-κB luciferase activity (Fig. 3C) and translocation of NF-κB from the cytosol to nuclei (Fig. 3D) compared to controls.

Id1 up-regulates cyclin D1 via NF-κB in keratinocytes

Immunohistochemical data demonstrated co-expression of NF-κB and cyclin D1 in basal cells of cholesteatoma

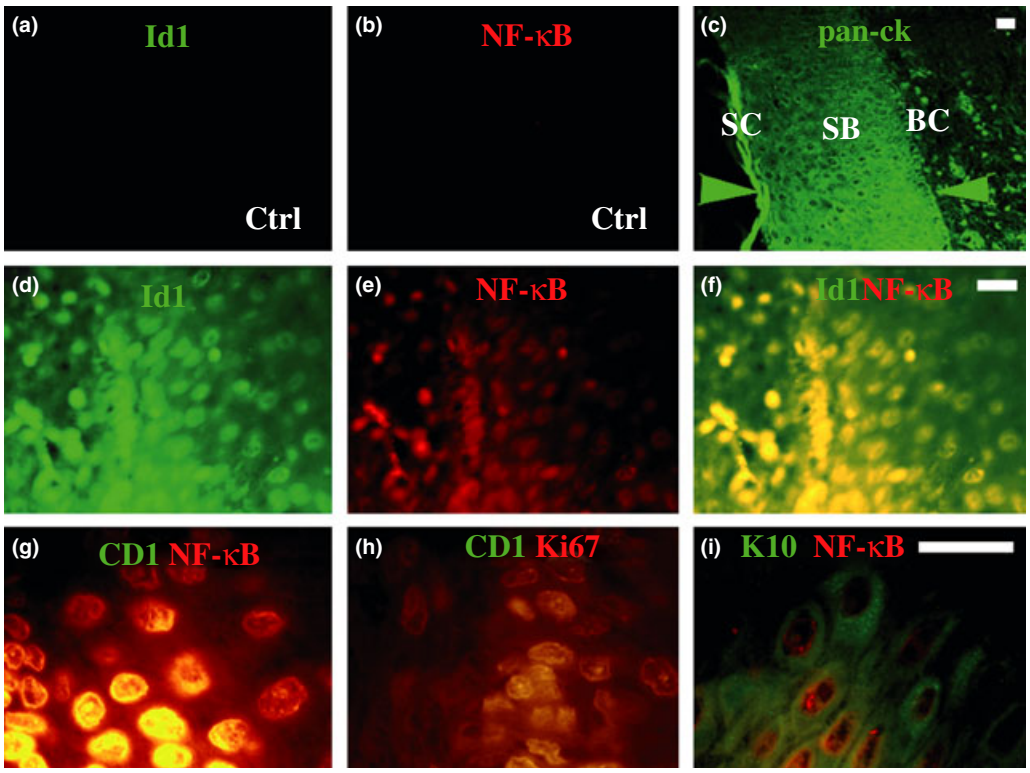

Figure 2. Id1, NF-κB, cyclin D1 and Ki67 are highly expressed in cholesteatoma tissues. Normal middle ear epithelium was negative for Id1 (a) and activated NF-κB (b), whereas cholesteatoma produced abundant pan-cytokeratins (pan-ck) in the basal cell (BC), and suprabasal (SB) and stratum corneum (SC) layers (c, between green arrowheads). Id1 (d) and activated NF-κB (e) were co-expressed in the basal cell (BC) layer (f) of cholesteatoma tissues. Similarly, activated NF-κB and cyclin D1 (g) as well as cyclin D1 and Ki67 (h) were co-expressed in the basal layer; while keratin 10 and NF-κB (i) were co-expressed in the suprabasal layer of cholesteatoma epithelium. Bar = 10 μm applying to the same row; Ctrl, control middle ear tissue (a and b); and cholesteatoma tissue (c–i).

tissues. To study whether Id1 promoted proliferation of keratinocytes *via* the NF-κB/cyclin D1 pathway and provided a link to aggressive growth of keratinocytes in cholesteatoma, we focused on this pathway using luciferase assays. Specifically, Rhek-1A cells were transfected with Id1, p65 and Id1+p65, respectively, co-transfected with wild-type or mutant cyclin D1/β-galactosidase reporters, and harvested for luciferase assays; Western blot analysis was carried out for evaluation of any potential link between NF-κB and cyclin D1 in the presence or absence of Id1. It was demonstrated that Id1 significantly increased luciferase activity of wild-type cyclin D1 reporters compared to empty vector (Fig. 4A, Id1 versus vec). Mutation of the NF-κB binding site at the cyclin D1 promoter fully abrogated Id1-induced cyclin D1 promoter activity (Fig. 4B, Id1 versus Id1+CDmt). This suggests that Id1 drives transcription of cyclin D1 *via* NF-κB. In addition, p65 significantly increased Id1-induced promoter activity of cyclin D1 (Id+p65 versus Id1), adding support to the notion that Id1 regulates cyclin D1 *via* NF-κB.

Id1 inhibited expression of p16^{Ink4a} but induced expression of keratin 10 via an NF-κB-dependent mechanism in keratinocytes

p16^{Ink4a} is an inhibitor of cyclin-dependent kinases, preventing cells from entering into the cell cycle (22). To study whether Id1 inhibit would expression of p16^{Ink4a} in keratinocytes, Id1 and empty vector were transfected into Rhek-1A cells and expression of p16^{Ink4a} and keratin 10 were evaluated by Western blot analysis. The data demonstrated that Id1 inhibited expression of p16^{Ink4a} (Fig. 5, middle row, Id1 versus vec) but increased expression of keratin 10 (Fig. 5, top row, Id1 versus vec). The same applied to p65 and Id1+p65. IκBαM, a dominant-negative inhibitor of NF-κB, inhibited expression of keratin 10 but rescued expression of p16^{Ink4a}. However, Id1 antagonized action of IκBαM on expression of keratin 10 and action of IκBαM on expression of p16^{Ink4a}. To study whether Id1-induced keratin 10 expression was also dependent upon NF-κB, Id1+p65 as well as Id1+ IκBαM were co-transfected into the cells. Results demonstrated that Id1+p65 strengthened expression of keratin 10 compared to p65

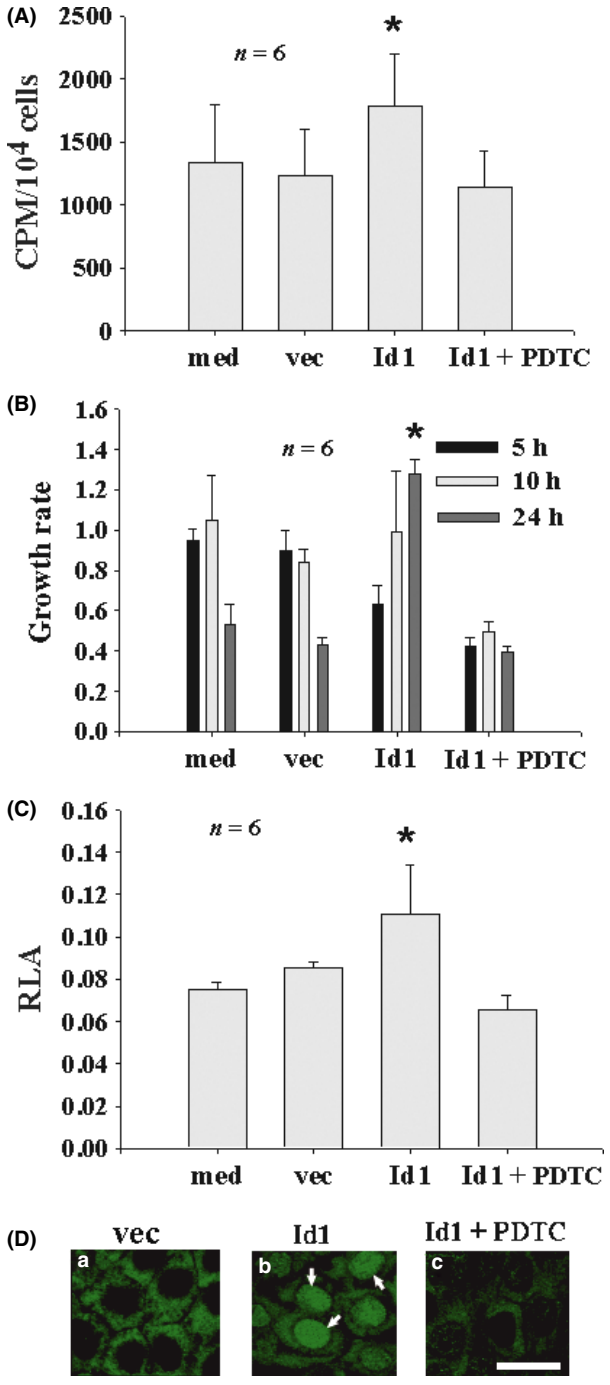

Figure 3. Id1 induces proliferation of keratinocytes *via* NF-κB *in vitro*. (A) Id1 significantly increased DNA synthesis (CPM, counts per minute) compared with vec, whereas PDTC eliminated the effect of Id on DNA synthesis of keratinocytes. (B) Id1 increased cell population growth rate in a time-dependent manner. At 24 h, Id1 significantly increased growth rate compared to vec. PDTC significantly inhibited the effect of Id1 on growth rate. Note that empty vector-transfected cells had high growth rate at 5 h but declined thereafter, whereas Id1-transfected cells had low growth rate at 5 h but increased thereafter. (C) Id1 significantly increased promoter activity of NF-κB (RLA, relative luciferase activity) compared to vec and PDTC eliminated the effect of Id1 on promoter activity of NF-κB. (D) Id1 increased NF-κB translocation into nuclei of Rhek-1A keratinocytes after 1 h transfection of Id1 compared to vec (arrows in panel b pointing to the nuclei of Rhek-1A keratinocytes, positive for activated NF-κB after staining with anti-activated NF-κB). Bar = 10 μm, applying to the same row in (D). med, medium alone as blank control; vec, empty vector as transfection control; $*P < 0.05$.

and Id1 alone, whereas IκBαM weakened expression of keratin 10 induced by Id1 compared to Id1.

Discussion

Id1 was originally recognized as a negative regulator of HLH DNA-binding proteins (5). Later, it was shown to be involved in neurogenesis and angiogenesis of tumour xenografts (23). In this study, we demonstrate for the first time that Id1 was involved in mucosal infectious diseases (1) and related to proliferative middle ear diseases.

As expected, Id1 was linked to the aggressive growth of acquired cholesteatoma through the Id1 → NF-κB → cyclin D1 → Ki67 signalling pathway (Fig. 1). First, Id1 activated NF-κB. NF-κB, in turn, activated transcription of cyclin D1. Cyclin D1 then increased progression of the cell cycles from G_0 to S phase. In addition, Id1 inhibited expression of $p16^{Ink4a}$, liberating cells from cell cycle inhibition. Id1 acting through NF-κB/cyclin D1/Ki67 signalling pathway provided an explanation of why middle ear infection is linked to cholesteatoma. However, this experiment was performed *in vitro* using immortalized keratinocytes and whether the above signalling pathway occurs in cholesteatoma matrix remains to be further studied. At present, it is not clear whether physical damage and trauma to the tympanic membrane trigger expression of the Id family.

Numerous mechanisms are proposed to underlie acquired cholesteatoma, all of them invoke inflammation of keratinocytes in some way. We have demonstrated in this study that activation of NF-κB is actively linked to cell proliferation in keratinocytes, which frequently originate from the external auditory canal skin. The molecular mechanism involves up-regulation of cyclin D1 and down-regulation of $p16^{Ink4a}$ in keratinocytes. The former is a cell cycle progression protein and the latter a cell cycle progression inhibitor. Through this mechanism, Id1 opens a window for keratinocytes to actively grow and proliferate. Our *in vitro* studies demonstrate that PDTC, an inhibitor of NF-κB, blocks Id1-induced proliferation of keratinocytes, indicating that Id1-induced cell proliferation is dependent on NF-κB.

Activity of the cyclin D/retinoblastoma (Rb) pathway leads to proliferation of cells (24). Cyclin D1, together with cyclin-dependent kinases 4/6 (cdk 4/6), overcomes the function of Rb protein that promotes cell cycle pro-

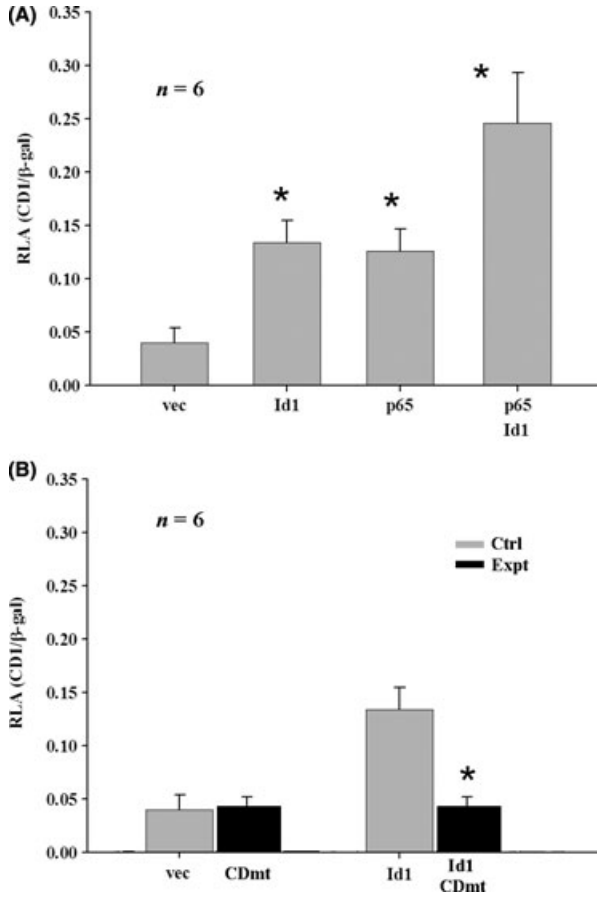

Figure 4. Id1 up-regulates cyclin D1 *via* NF-κB in keratinocytes *in vitro*. (A) Id1 significantly increased luciferase activity of cyclin D1 in Rhek-1A cells (Id1 versus vec); p65 further increased Id1-induced action (Id1+p65 versus Id1); p65 itself significantly increased luciferase activity of cyclin D1 (p65 versus vec); and Id1+p65 significantly increased luciferase activity of cyclin D1 compared with Id1 or p65 alone (Id1+p65 versus Id1 or p65). (B) Mutation of the NF-κB-binding site at the promoter of cyclin D1 (CDmt) fully abrogated the Id1-indued cyclin D1 promoter activity (Id1 versus Id1+CDmt); and CDmt itself did not increase or decrease luciferase activity (CDmt versus vec). *$P < 0.05$ when compared with vec in (A) or Id1 in (B).

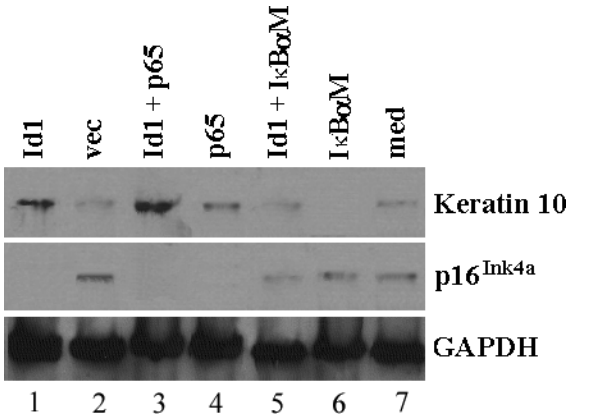

Figure 5. Id1 positively regulates expression of keratin 10 *via* NF-κB but negatively regulates expression of p16^{Ink4a} in keratinocytes *in vitro*. Western blot analysis demonstrated that Id1 increased expression of keratin 10 (lane 1, top) compared with its control (lane 2, top). p65 further increased Id1-induced expression of keratin 10 (lane 3 versus lane 1, top) but IκBαM abrogated Id1-induced expression of keratin 10 (lane 5 versus lane 1, top). p65 itself also increased expression of keratin 10 (lane 4) compared with empty vector (lane 2, top). Id1, p65 and Id1+p65 inhibited expression of p16^{Ink4a} (lanes 1, 3 and 4, middle) compared with empty vector (lane 2, middle) in Rhek-1A cells. On the contrary, IκBαM increased expression of p16^{Ink4a} (lane 6, middle) but Id1 overcame the action of IκBαM on expression of p16^{Ink4a} (lane 5, middle). med, blank control (without any transfection). GAPDH, glyceraldehyde 3-phosphate dehydrogenase (loading control).

gression. It is generally accepted that normal Rb function must be removed, one way or another, for a cell to divide and down-regulation of p16^{Ink4a} is one of the mechanisms for removing Rb function. Cyclin D1 is a well-established positive regulator of early cell cycle progression, that is, transition from G_0/G_1 to S phase (25) through phosphorylation of Rb and dissociation of E2F (a checkpoint protein for S phase entry). Up-regulation of cyclin D1 and removal of p16^{Ink4a} inhibition in Rhek-1A cells by Id1, potentiates population growth and proliferation of keratinocytes and in part explains the behaviour of keratinocytes in cholesteatoma – aggressive growth (26–28) that destroys the ossicular chain and temporal bone and causes serious complications such as deafness and intracranial lesions. Id1-induced cyclin D1 up-regulation and p16^{Ink4a} down-regulation may represent a disease mechanism for cholesteatoma epithelial growth under chronic inflammatory conditions.

Activation of NF-κB not only increases proliferation of keratinocytes but also up-regulates production of keratins. Keratin 10 is a product of mature keratinocytes (29–31) and is highly expressed in the external auditory canal skin. Abundant expression of keratin 10 suggests that keratinocytes in middle ear cholesteatoma originated from the external auditory canal epidermis possibly *via* a migration process under chronic inflammatory conditions (32). Biologically, cell migration is coupled with cell proliferation. Our data indicate that Id1 regulates NF-κB, and NF-κB, in turn, up-regulates expression of keratin 10, which represents a pathological mechanism for accumulation of the onion peel-like substances in cholesteatoma. p65 strengthens this process, whereas IκBαM attenuates it.

It is noted that NF-κB at the suprabasal layer and above may not be related to cell population growth and proliferation because cells at the suprabasal layer and above do not grow and proliferate but commit to keratin production. Earlier studies point out that NF-κB at the suprabasal layer and above may be related to survival and protection of cells from apoptosis (33–35). Why Id1-induced NF-κB in the basal layer leads to cell prolifer-

ation, while NF-κB alone beyond the basal layer results in cell survival and resistance to apoptosis remains an interesting question. It is also not clear from this study how Id1 inhibits expression of $p16^{Ink4a}$. It is warranted to address these questions in the future because of their importance in pathogenesis of cholesteatoma and physiology of the related epidermis.

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