Tumor-Selective Induction of Apoptosis and the Small-Molecule Immune Response Modifier Imiquimod

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Background: The incidence of nonmelanoma skin cancer, basal cell carcinomas (BCCs), and squamous cell carcinomas (SCCs) is increasing, representing a major medical and economic problem. Imiquimod, a topical small-molecule immune response modifier, has shown efficacy toward BCC and actinic keratoses in clinical trials. Imiquimod acts both indirectly, via cytokine-mediated stimulation of cellular immune responses, and directly, through unknown mechanisms against tumor cells. We examined the mechanism by which imiquimod induces apoptosis in cancer cells. Methods: Apoptosis was assessed by enzyme-linked immunosorbent assay, western blot analysis, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays in five SCC cell lines, HaCaT cells (a spontaneously immortalized human keratinocyte cell line), and normal keratinocytes treated with imiquimod, with its analog resiquimod, or with neither. Expression of death receptors, caspases, and cytochrome c in the apoptotic signaling cascade was analyzed using western blot and flow cytometric analyses. The functional relevance of imiquimod-induced cytochrome c release was assessed by transfection of HaCaT cells with Bcl-2. Apoptosis in BCCs in vivo was assessed by TUNEL assays of imiquimod-treated and untreated tumors from three patients. Differences between treated and untreated cells and tumors were determined using a two-tailed Student’s t test. Results: Imiquimod, but not resiquimod, induced apoptosis in all SCC cell lines and HaCaT cells. This induction involved activation of several caspases and Bcl-2–dependent cytosolic translocation of cytochrome c but was independent of the membrane-bound death receptors Fas, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)-R1–R4 receptors, and tumor necrosis factor-R1 and -R2 receptors. Topical application of imiquimod to BCC tumors in vivo induced apoptosis. Conclusion: Imiquimod has the potential to induce apoptosis in skin cancer cells, possibly by circumventing mechanisms developed by malignant tumors to resist apoptotic signals. [J Natl Cancer Inst 2003;95:1138–49]
To evaluate whether imidazoquinolines directly affected tumor cells, molecular mechanisms of induction of apoptosis by these compounds were of great interest. Two major routes have been identified through which cytostatic drugs may induce apoptosis. One involves activation of membrane-bound death receptor systems, such as CD95 (i.e., Fas/APO-1), tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL), and the other is dependent on direct mitochondrial cytochrome c release (39–41). Thus, at least three major and not mutually exclusive hypotheses can be delineated concerning the molecular mechanisms involved in imiquimod-induced apoptosis of epithelial tumor cells. First, imiquimod could act directly on membrane-bound death receptors, thus initiating the apoptotic signal transduction cascade triggered by one or more of these death receptors. Second, as described for ceramides (42), imiquimod could affect molecules downstream of the death receptors triggering the caspase cascade and subsequent cell death, thus bypassing the membrane-bound receptors. Third, imiquimod could influence Bcl-2-dependent mitochondrial cytochrome c release, leading to activation of caspase-9 and subsequent cell death.

Given that some tumors have developed an aberrant regulation within death receptor–mediated pathways that allows them to escape from immune surveillance (43), bypassing these pathways by delivering direct apoptotic signals through antineoplastic compounds may be an extremely important strategy for treating such tumors (44). In this study, we have evaluated the pro-apoptotic activity of imiquimod 

**Materials and Methods**

**Cells and Culture Conditions**

Primary cultures of normal human keratinocytes were established from surgical specimens of human foreskin or skin from breast reductions and cultured as previously described (45,46). The cell lines SCC12, SCC13, and SCC15 (gifts from J. G. Rheinwald, Harvard Medical School, Boston, MA) and SCL-1 and SCL-2 (provided by N. E. Fusenig, German Cancer Research Center, Heidelberg, Germany) are human cell lines derived from SCCs of the skin (SCC13, SCC15, SCL-1, SCL-2) and the oral cavity (SCC12). HaCaT (gift from N. E. Fusenig) is a spontaneously immortalized human keratinocyte cell line (47). For apoptosis experiments, all epithelial cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin at 100 U/mL, streptomycin at 100 U/mL, and 1% nonessential amino acids (all from Life Technologies, Karlsruhe, Germany).

**Overexpression of Bcl-2 in HaCaT Cells**

To assess the role of Bcl-2 in imiquimod-induced apoptosis, HaCaT transfectants stably overexpressing functional murine Bcl-2 were generated as previously described (42). Transgene-containing cells were selected using genetin at 0.6 mg/mL (G418; Life Technologies), and stable expression of murine Bcl-2 was confirmed by western blot analysis.

**Antibodies**

Murine monoclonal antibodies (MAbs) specific for human CD95 (i.e., Fas/APO-1, clone CH-11 murine immunoglobulin M [IgM]; Immunotech, Marseille, France, and clone Z4 murine IgG; Chemicon, Hofheim, Germany) were used in functional assays of CD95 (i.e., activation and inhibition, respectively) at final concentrations of 1 μg/mL. Anti-TRAIL-R1 (DR4) affinity-purified goat IgG, anti-TRAIL-R2 (DR5) affinity-purified goat IgG, anti-TRAIL-R3 (DcR1) affinity-purified goat IgG, and anti-TRAIL-R4 (DcR2) affinity-purified goat IgG (all obtained from R&D Systems, Wiesbaden, Germany) were used at concentrations of 50 ng/mL to assess the functional status of TRAIL receptors R1–R4, respectively. Murine MAB225 (mouse IgG1, clone 16803) and MAB226 (mouse IgG2A, clone 222221.311) (both obtained from R&D Systems) were used at concentrations of 3 μg/mL to assess the functional status of TNF receptors TNF-R1 and TNF-R2, respectively. Anti-murine Bcl-2 MAbs (clone 100/D5, 1:1000 dilution; Novocastra, Dossenheim, Germany), rabbit anti-caspase-3 polyclonal antibodies (clone CPP32, 1:2000 dilution; BD Pharmingen, Hamburg, Germany), and murine anti-cytochrome c MAbs (clone 7H8.2C12, 1:1000 dilution; Pharmingen) were used in western blot analyses. Isotype-matched MAbs (murine IgG1: MAB002, clone 11711; murine IgG2A: MAB003, clone 20102; rabbit IgG: AB-105-C; all obtained from R&D Systems, used at a final concentration of 3 μg/mL) and/or normal goat serum (purchased from Dako, Hamburg, Germany) were used as negative controls.

**Determination of the Subcellular Distribution of Imiquimod and Resiquimod by High-Performance Thin-Layer Chromatography**

To assess the subcellular distribution of imiquimod and resiquimod in the cytosol and the membrane fraction, cultures of HaCaT cells in normal culture medium were incubated with imiquimod (50 μg/mL), resiquimod (50 μg/mL) (both compounds donated by 3M Pharmaceuticals, St. Paul, MN), or normal medium alone for 24 hours at 37°C and 5% CO2 in a humidified incubator. Cells were then washed three times with phosphate-buffered saline (PBS), harvested, lysed in a hypotonic buffer (containing 20 mM HEPES buffer, 10 mM KCl, 2 mM MgCl2, 1 mM EDTA [pH 7.4]) for 15 minutes on ice, and homogenized by 20 passages through a syringe equipped with a 28-gauge needle. The cytosolic and the membrane fractions were separated by centrifugation at 16000g for 10 minutes at 4°C; the centrifugation step was repeated on the cytosolic fraction. The cytosolic fractions, membrane fractions, and culture medium (all 2-mL aliquots) were then lyophilized overnight using a UniEquip system (UniEquip, Martinsried, Germany), followed by extraction (detailed below) of the compounds for subsequent detection by high-performance thin-layer chromatography (HPTLC).

Given that no data regarding subcellular distribution of imiquimod and resiquimod were available, we established experimental conditions under which both compounds could be extracted from the lyophilized samples and detected by HPTLC. In a series of experiments, we established that imiquimod was reliably extracted and separated from cellular components using 100 μL of chloroform/methanol (1:1, vol/vol) as a solvent, whereas the best experimental conditions for extracting resiquimod used 100 μL of chloroform as a solvent.
Standards of purified imiquimod and resiquimod (20 μg/mL of a 50-μg/mL solution) and extracts of the cellular fractions from each sample (75 μL) were applied to silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) using an HPTLC applicator (Linomat III; Camag, Berlin, Germany) and were separated by placing the plates in a mixture of 2-propanol (47.75%), n-heptane (47.75%), chloroform (2.5%), acetone (1.0%), acetic acid (0.50%), and water (0.50%). Imiquimod and resiquimod were then visualized by placing the plates in 5% H2SO4 and heating for 15 minutes at 180°C. Based on these experiments, the calculated retardation factor (Rf) value for imiquimod was 0.71 (95% confidence interval [CI] = 0.70 to 0.72), whereas resiquimod resolved in a broader band resulting in an Rf value of approximately 0.68 (95% CI = 0.67 to 0.69). Rf values were derived from three independent experiments.

Flow Cytometric Analysis

To determine surface expression of membrane-bound death receptors, adherent SCC or HaCaT cells were detached from the tissue-culture plates using 0.25% trypsin/0.02% EDTA in PBS. Cells were then incubated with primary MAb specific to the protein of interest (10 μg/mL) at 4°C for 45 minutes, washed three times in PBS, and incubated with phycoerythrin-labeled goat anti-mouse IgG for 45 minutes at 4°C. The cells were again washed three times in PBS, fixed in 2% paraformaldehyde, and analyzed using a FACScan fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany) in conjunction with CellQuest software (Becton Dickinson, Heidelberg, Germany). A Bradford assay to determine the protein concentration in the lysates was performed according to the manufacturer’s instructions. Briefly, normal keratinocytes, SCC cells, and HaCaT cells were incubated with pro-apoptotic stimuli (i.e., imidazoquinolines, CD95-activating MAb, TRAIL, TNF), harvested (both adherent and detached cells) in PBS containing 0.25% trypsin/0.02% EDTA, lysed in 1% Triton X-100 for 15 minutes at 4°C, and centrifuged at 10000g for 10 minutes at 4°C. Equal amounts of the resulting supernatant were added to 96-well microtiter plates coated with anti-histone antibodies (murine IgG, clone H11–4, according to the manufacturer instructions) and incubated for 90 minutes at room temperature. After three washes in PBS, the secondary antibody (anti-DNA peroxidase antibody; murine IgG, clone MCA-33, according to the manufacturer instructions) was applied, and the samples were further incubated for 90 minutes at room temperature. The samples were then washed three times in PBS. The substrate reaction [i.e., cleavage of the (2,2'-Azinodi-[3-ethylbenzthiazolin-sulfonate(6) (ABTS) chromogenic compound by the MCA-33–conjugated peroxidase], resulting in a green color, was quantitated (i.e., measured against substrate solution) using an ELISA reader at 405 nm. All experiments were performed in duplicate and were repeated at least three times.

Western Blot Analysis

To evaluate total expression of death receptors and intracellular apoptosis-related proteins, cultured SCC or HaCaT cells were solubilized in 1% Triton X-100 (Sigma Chemicals, Deisenhofen, Germany) supplemented with Complete Protease Inhibitor (one tablet per 50 mL; Boehringer Mannheim, Mannheim, Germany). A Bradford assay to determine the protein concentrations in the lysates was performed according to the manufacturer’s instructions (BioRad, München, Germany). Equal amounts of protein from each cell lysate (20 μg of total protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred by electroblotting to a nitrocellulose membrane. Proteins of interest were then detected by probing with their specific MAbS (at concentrations specified above) followed by incubation in peroxidase-labeled goat anti-mouse IgG (3 μg/mL). Antibody-reactive protein bands were visualized with the use of an enhanced chemiluminescence reaction system (Amersham Pharmacia, Buckinghamshire, U.K.). Protein bands were quantitated densitometrically using a Fluor-S MultiImager (Bio-Rad). Each experiment was performed at least three times.

Cytotoxicity Assays

To assess whether cell death was a result of apoptosis or necrosis, cytotoxicity (i.e., necrosis) was determined using a lactate dehydrogenase (LDH)-release detection kit according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). Briefly, cultured normal keratinocytes, SCC cells, and HaCaT cells were incubated with normal culture medium, imiquimod, or resiquimod at concentrations ranging from 0 to 50 μg/mL for time periods ranging from 0 to 48 hours. Culture supernatants were then cleared of debris by centrifugation at 300g for 5 minutes at room temperature. An aliquot of the supernatant (100 μL) was then transferred to a 96-well microtiter plate, and the LDH activity was determined by measuring the generation of the formazan salt chromagen in an enzyme-linked immunosorbent assay (ELISA) reader at 492 nm. Each experiment was performed at least three times.

DNA Fragmentation Assay

For detection of cytoplasmic histone–associated DNA fragments, the Cell Death Detection ELISA (Roche Diagnostics) was used according to the manufacturer’s instructions. Briefly, normal keratinocytes, SCC cells, and HaCaT cells were incubated with pro-apoptotic stimuli (i.e., imidazoquinolines, CD95-activating MAb, TRAIL, TNF), harvested (both adherent and detached cells) in PBS containing 0.25% trypsin/0.02% EDTA, lysed in 1% Triton X-100 for 15 minutes at 4°C, and centrifuged at 10000g for 10 minutes at 4°C. Equal amounts of the resulting supernatant were added to 96-well microtiter plates coated with anti-histone antibodies (murine IgG, clone H11–4, according to the manufacturer instructions) and incubated for 90 minutes at room temperature. After three washes in PBS, the secondary antibody (anti-DNA peroxidase antibody; murine IgG, clone MCA-33, according to the manufacturer instructions) was applied, and the samples were further incubated for 90 minutes at room temperature. The samples were then washed three times in PBS. The substrate reaction [i.e., cleavage of the (2,2'-Azinodi-[3-ethylbenzthiazolin-sulfonate(6) (ABTS) chromogenic compound by the MCA-33–conjugated peroxidase], resulting in a green color, was quantitated (i.e., measured against substrate solution) using an ELISA reader at 405 nm. All experiments were performed in duplicate and were repeated at least three times.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

Cultures of HaCaT cells and SCL-1 cells were incubated with normal culture medium, imiquimod (50 μg/mL), or resiquimod (50 μg/mL). After 24 hours, the cells were detached from the culture flask using 0.25% trypsin and fixed on microscope slides using 4% formalin for 10 minutes at room temperature. Apoptotic cells were detected using a modified terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit (DermaTACS: R&D Systems). Briefly, slides were treated with Proteinase K (1 μg in 50 μL of DNase-free H2O) to ensure that the DNA fragments were accessible to the labeling enzymes, and the activity of endogenous peroxidase was quenched using 3% H2O2 in methanol. DNA fragmentation in individual apoptotic cells was then detected by labeling with terminal deoxynucleotidyl transferase (TdT) and the thymidine analog bromodesoxyuridine (BrdU). The reaction was stopped by the addition of EDTA at a final concentration of 10 mM, and the cells were washed three times in PBS to remove any unincorporated BrdU. Incorporated BrdU was detected by a specific and sensitive biotinylated anti-BrdU antibody in combination with a streptavidin–peroxidase conjugate (R&D Systems), according to the manufacturer’s instructions. Briefly, cells were incubated with the anti-BrdU MAb at 37°C for 30 minutes, followed by three washes in PBS/Tween 20. The slides were then incubated with the streptavidin–peroxidase conjugate at
room temperature for 10 minutes, followed by two washes in PBS and one wash in H2O. Labeled cells were visualized with TACS Blue Label (R&D Systems), which generates an intense blue staining in cells with DNA fragmentation. In each experiment, one aliquot of cells was treated with TACS nuclease (to generate fragmentation of DNA in all cells, i.e., a positive control), and one aliquot of cells was used in which TdT had been omitted (i.e., a negative control). DNA fragmentation (i.e., TUNEL-positive cells) was quantitated microscopically using an AxioVision 2 Plus (Zeiss, Jena, Germany) and the Axiocapture 3.0 image analysis software (Zeiss). The total number of TUNEL-positive cells was quantitated by assessing a total of 1000 cells per culture and calculating the ratio of apoptotic cells to total cells. For each condition, at least three independent experiments were performed.

Inhibition of Caspase Activation

SCL-1 and SCC13 tumor cells and HaCaT cells that were induced to undergo apoptosis were cultured in the presence or absence of caspase inhibitors that bind to the active site of their respective protease. The following fluoromethyl ketone (FMK)-linked caspase inhibitors were used because they exhibit enhanced cellular permeability for use in *in vitro* cell cultures: Z-VAD-FMK (general caspase inhibitor), Z-WEHD-ZMK (caspase-1 inhibitor), Z-DEVd-ZMK (caspase-3 inhibitor), Z-YVAD-ZMK (caspase-4 inhibitor), Z-VEID-ZMK (caspase-6 inhibitor), Z-IETD-ZMK (caspase-8 inhibitor), Z-LEHD-ZMK (caspase-9 inhibitor), Z-AEVD-ZMK (caspase-10 inhibitor), Z-LEED-ZMK (caspase-13 inhibitor) (all obtained from R&D Systems). Inhibitors were dissolved in dimethyl sulfoxide at a concentration of 10 μM and diluted with normal culture medium to a final concentration of 10 nM. Cells were preincubated with caspase inhibitors for 10 minutes at 37 °C before adding either imiquimod (50 μg/mL) or CH-11 MAb (1 μg/mL) for 24 hours at 37 °C. Control cells were incubated in dimethyl sulfoxide (i.e., vehicle) diluted 1:1000 in culture medium for 24 hours at 37 °C and 5% CO2.

Subcellular Fractionation

Cytosolic and mitochondrial extracts were prepared as described previously (48). Briefly, HaCaT, SCL-1, and SCC13 cells were detached from culture dishes using 0.25% trypsin, harvested in PBS, lysed in hypotonic buffer containing Complete Protease Inhibitor, and incubated on ice for 15 minutes. The cells were then homogenized by repeated passage through a 28-gauge needle and centrifuged at 10000g for 10 minutes at 4 °C. Cytosolic fractions were collected from the supernatants, and the mitochondrial pellets were solubilized in lysis buffer (1% Triton X-100 containing Complete Protease Inhibitor). Western blot analyses of cytosolic and mitochondrial fractions were performed as previously described (48).

Induction of Apoptosis In Vitro

Subconfluent cultures of HaCaT, SCL-1, and SCC13 cells were incubated for up to 48 hours with either imiquimod or resiquimod (at concentrations ranging from 1 to 50 μg/mL, diluted in normal culture medium at 37 °C). To stimulate the TRAIL receptor system, TRAIL at 10 ng/mL (R&D Systems) was added to the cultures. TRAIL receptors R1–R4 were blocked using their respective function-blocking antibodies (all at 50 ng/mL). To stimulate the TNF receptor system, TNF-α (1 ng/mL) was added to the cultures. TNF receptors TNF-R1 and TNF-R2 were blocked using their respective function-blocking MAbs (both at 3 μg/mL).

**Induction and Detection of Apoptosis In Vivo**

Three patients suffering from multiple superficial BCCs of the trunk were treated with a topical application of imiquimod (i.e., Aldara 5% cream) after providing written informed consent. Each patient had one of their tumors treated with imiquimod daily for 12 hours (i.e., the cream was left on under an occlusive dressing) on 4 consecutive days. Punch biopsies (6 mm) were then taken from the treated tumor and from an untreated tumor. Tissue specimens were fixed in 4% formalin to prevent the loss of low-molecular-weight DNA fragments, embedded in paraffin, and sectioned (5 μm). Paraffin-embedded sections from treated and untreated tumors were subjected to apoptosis detection by a modified TUNEL method (detailed above).

**Statistical Analyses**

All statistical analyses were performed using Excel software (Microsoft, Redmond, WA). Data are presented as means and 95% CIs. Differences between treated and untreated conditions were determined using a two-tailed Student’s *t* test. *P* values less than .05 were considered statistically significant, and all statistical tests used were two-sided.

**RESULTS**

**Effect of Imiquimod and Resiquimod on Normal Keratinocytes and Epithelial Tumor Cells**

To determine the biologic activity of imidazoquinolines on nonmelanoma skin cancer cells, five keratinocyte-derived epithelial tumor cell lines, the spontaneously immortalized keratinocyte cell line HaCaT, and normal keratinocytes were cultured in normal culture medium or culture medium supplemented with imiquimod or resiquimod (Fig. 1, A) at concentrations up to 50 μg/mL, which is 1000-fold below the marketed formulation of Aldara 5% cream (50 mg/g). Diminished cell counts were consistently observed in the tumor cell lines in a concentration-dependent manner. In the SCC (i.e., SCL-1, SCC-2, SCC12, SCC13, and SCC15) and HaCaT (47) cell cultures, treatment with imiquimod reduced the total cell counts by 40%–70% compared with vehicle-treated cell cultures (Fig. 1, B, left panel; HaCaT, *P* = .038; SCL-1, *P* = .012; and SCC13, *P* = .020). Reduction in cell count was considerably less pronounced when the cells were cultured in the presence of resiquimod (Fig. 1, B, right panel). The effect of imiquimod on reducing cell counts was surprising because imiquimod had not previously been found to exert direct antitumoral effects at lower concentrations (i.e., up to 10 μg/mL) (28,38). Therefore, we assessed the underlying molecular mechanisms of this imiquimod-induced reduction of cell counts.

To distinguish between necrosis and apoptosis, cytotoxic effects of both imiquimod and resiquimod on normal keratinocytes and four keratinocyte-derived tumor cell lines (i.e., SCL-1, SCL-2, SCC13, and HaCaT) were assessed using the LDH release method. Consistent with previous reports (19,31,32), neither imiquimod nor resiquimod exerted cytotoxic effects when
Fig. 1. Imiquimod and tumor-selective apoptosis in squamous cell carcinoma (SCC) cell lines. A) Chemical structures of imiquimod (1-(2-methylpropyl)piperidine-1-1H-imidazo[4,5-c]quinolin-4-anine; upper panel) and resiquimod (4-amino-\(\alpha\)-dimethyl-2-ethoxymethyl-1H-imidazo[4,5-c]quinoline-1-ethanol; lower panel). B) Normal keratinocytes (KC) (filled squares), SCC13 cells (filled circles), SCL-1 cells (filled triangles), and HaCaT cells (a spontaneously immortalized human keratinocyte cell line; filled diamonds) were cultured in the presence of imiquimod (left panel) or resiquimod (right panel) at the indicated concentrations. Data are shown as means and 95% CIs of three independent experiments. C) Normal keratinocytes (nKC) (filled symbols) and SCL-1 (open symbols) were cultured in the presence of imiquimod (left panel) or resiquimod (right panel) at the indicated concentrations. Cytotoxicity (i.e., necrosis) was determined using a lactate dehydrogenase (LDH) assay after 12 hours (filled and open squares), 24 hours (filled and open circles), and 48 hours (filled and open diamonds) of incubation. The gray line in the left panel represents the mean of all values from one experiment, which is representative of three independent experiments showing similar results. Data are shown as the means and 95% CIs of three independent experiments. D) Normal keratinocytes (nKC) (filled squares), SCC13 (filled circles), SCL-1 (filled triangles), and HaCaT cells (filled diamonds) were cultured in the presence of imiquimod (left panel) or resiquimod (right panel) at the concentrations indicated. Apoptosis was assessed by determining histone-bound DNA fragments 24 hours after incubation. Data are shown as means and 95% CIs of three independent cultures in one representative experiment. The experiment shown is representative of five independent experiments (each with three cultures) showing similar results. E) HaCaT cells were incubated in normal culture medium (left panel) or in medium containing resiquimod (middle panel) or imiquimod (right panel). Apoptosis was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay after 24 hours of incubation. Apoptotic (i.e., TUNEL-positive) cells are visualized by intensive blue staining. Inset shows a high-power magnification of an apoptotic cell. F) HaCaT cells were incubated in normal culture medium (control; filled bar) or in medium containing imiquimod (hatched bar) or resiquimod (open bar). TUNEL assays were performed after 48 hours of incubation, and the number of apoptotic cells was quantitated microscopically. Data are shown as means and 95% CIs from three independent cultures for each condition. * indicates \(P = .004\) comparing imiquimod-treated cultures with resiquimod-treated cultures and \(P = .009\) comparing imiquimod-treated cultures with untreated control cultures.

Applied at concentrations ranging from 0 to 50 \(\mu\)g/mL (Fig. 1, C; SCC-1 tumor cells and normal keratinocytes are depicted). In contrast, when apoptosis was assessed, imiquimod exerted marked pro-apoptotic effects in three transformed cell lines in a dose-dependent manner, with apoptotic DNA fragmentation 150%–450% higher than the apoptotic DNA fragmentation in control cultures; in some experiments, the increase in fragmentation was as much as 750% higher than that in control cultures \(P < .001\) (Fig. 1, D, left panel). The pro-apoptotic effect of imiquimod on normal human keratinocytes was, however, much weaker, only 5%–60% higher than that in control cultures at its highest concentration (Fig. 1, D, right panel).

To confirm the pro-apoptotic effect of imiquimod on epithelial tumor cells by a second method, TUNEL assays were performed using cultured HaCaT cells incubated with normal culture medium or culture medium supplemented with resiquimod or imiquimod for 24 hours (Fig. 1, E). In control cultures and in resiquimod-treated cultures, the percentages of apoptotic (i.e., TUNEL-positive) cells were 1.6% (95% CI = 1.0% to 2.2%) and 1.1% (95% CI = 0.7% to 1.5%), respectively. In contrast, in cultures incubated with imiquimod for 24 hours, 17.3% (95% CI = 15.2% to 19.4%; \(P = .004\) compared with resiquimod-treated cultures and \(P = .009\) compared with untreated control cultures) of the cells were apoptotic (Fig. 1, F). These results suggest that the diminished in vitro cell counts in resiquimod-treated cultures of epithelial tumor cells may be due, at least in part, to apoptosis induced by imiquimod. In contrast, resiquimod did not exert any statistically significant pro-apoptotic effects (Fig. 1, D–F); therefore, it was used as a negative control in all subsequent experiments.

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**Imiquimod-Induced Apoptosis and Membrane-Bound Death Receptors**

To elucidate the mechanism of imiquimod-induced apoptosis, we first determined whether imiquimod acts directly on membrane-bound death receptors. To evaluate the effects of imiquimod on the Fas/APO-1 receptor system, CD95 (Fas/APO-1) expression in SCC cell lines and normal keratinocytes was examined following culture with imiquimod. Alterations in CD95 expression could not be detected by western blot (Fig. 2, A) or by flow cytometric analysis (Fig. 2, B). Hence, to test whether imiquimod might activate CD95 without altering its expression, we compared apoptosis induced by imiquimod to apoptosis induced by the CD95-activating CH-11 MAb. Apoptosis of SCL-1 tumor cells was increased by approximately 270%–280% by both imiquimod (95% CI = 260.91% to 278.69%) and the CH-11 MAb (95% CI = 267.12% to 282.08%) (P = .003 and P = .002 compared with vehicle controls, respectively; Fig. 2, C). However, when SCL-1 tumor cells were incubated with the CD95-directed function-blocking ZB4 MAb, CH-11/CD95-induced apoptosis was diminished to the same level as that in vehicle-treated control cultures, whereas imiquimod-induced apoptosis was not affected (P = .002 comparing CH-11/ZB4-treated cells with imiquimod/ZB4-treated cells; Fig. 2, C). These results suggest that imiquimod-induced apoptosis is independent of CD95 receptor activation.

In the next series of experiments, the effects of imiquimod on the TRAIL receptor system were assessed by determining TRAIL-R1–4 receptor expression in SCC cell lines after treatment with imiquimod. TRAIL receptors R1–R4 were expressed by SCL-1, SCC13, and HaCaT cells at moderate levels (mean fluorescence intensity was between 30 and 80, data not shown). Although TRAIL bound to all four TRAIL receptors (R1–4), only TRAIL-R1 (DR4) and TRAIL-R2 (DR5) have been shown
to confer intracellular pro-apoptotic signals via activation of instigator caspases (e.g., caspase-8) (49). In contrast, TRAIL-R3 and TRAIL-R4 have been termed decoy receptors because they do not transmit pro-apoptotic signals and may even have anti-apoptotic effects due to the lack of (or nonfunctional) cytoplasmic death domains (50,51). Similar to the pro-apoptotic effect observed with the CD95-stimulating MAb CH-11, activation of TRAIL receptors in SCL-1 and HaCaT cells by TRAIL resulted in a marked induction of apoptosis to 329% (95% CI = 325.2% to 333.4%) of the apoptosis found in untreated cultures (P = .001, Fig. 2, D). The function-blocking antibodies against TRAIL-R1 and TRAIL-R2 statistically significantly diminished the pro-apoptotic activity of TRAIL by 39% (95% CI = 37.2% to 40.8%) and 43% (95% CI = 40.7% to 45.3%), respectively (P = .033 and P = .025 compared with TRAIL-treated cultures, respectively; Fig. 2, D). As expected, blocking of TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) did not inhibit the pro-apoptotic effect of TRAIL activation (Fig. 2, D). In contrast, imiquimod-induced apoptosis in SCL-1 or HaCaT cells was not inhibited by blocking TRAIL receptors (Fig. 2, D), suggesting that imiquimod-induced apoptosis is independent of the TRAIL receptor system.

In a final series of death receptor experiments, we evaluated the effect of inhibiting the TNF receptor system (R1–2) on imiquimod-induced apoptosis in SCL-1 and HaCaT cells. TNF-R1 expression was almost absent in both cell lines, whereas TNF-R2 was expressed at low levels in both (mean fluorescence intensity was approximately 20; data not shown). Consistent with this finding, incubation of the cells with TNF-α did not result in a detectable induction of apoptosis (Fig. 2, E). In addition, function-blocking antibodies to TNF-R1 and TNF-R2 did not affect imiquimod-induced apoptosis (Fig. 2, E).

Overall, blocking the functions of membrane-bound death receptors did not affect the pro-apoptotic activity of imiquimod; however, it did prevent apoptosis induced by receptor-specific stimulation. Therefore, these results suggest that imiquimod can bypass the signal transduction pathways initiated by membrane-bound death receptors in keratinocyte-derived tumor cell lines.

**Imiquimod-Induced Apoptosis and Caspase-3**

Because activation of pro-caspase-3 to functionally active caspase-3 is one of the final “executing” molecular steps leading to apoptosis (49), we assessed whether imiquimod affects activation of pro-caspase-3 by incubating SCL-1, SCC13, and HaCaT cells in normal culture medium or culture medium supplemented with imiquimod or resiquimod. Cells were then lysed, and levels of pro-caspase-3 and activated caspase-3 were evaluated by western blot analysis. Although resiquimod did not lead to activation of pro-caspase-3, treatment with imiquimod resulted in the appearance of a strong signal for active caspase-3 (lower band). SCL-1 tumor cells were cultured in normal medium (control; open bar) or in medium containing imiquimod (filled bar) or combinations of imiquimod and specific oligopeptide inhibitors of caspases as indicated (hatched bars) for 24 hours; VAD inhibits all caspases through binding to a shared functional motif. Apoptosis was assessed by determining histone-bound DNA fragments and is expressed as means and 95% confidence intervals (relative to the control [untreated cells]). *P = .001 for VAD, P = .001 for caspase-3, P = .036 for caspase-4, P = .003 for caspase-6, P <.001 for caspase-8 and -9, and P = .002 for caspase-10, all compared with imiquimod-treated cultures. The experiment shown is representative of three independent experiments showing similar results. The black vertical line represents the average apoptosis in untreated cells.

**Imiquimod-Induced Apoptosis and Caspase Activation**

Because imiquimod induced activation of caspase-3, we sought to assess the involvement of other caspases in the process of imiquimod-induced apoptosis by using potent specific oligopeptide inhibitors that are modeled after the substrate recognition sequences of a variety of caspases. Caspases exhibit highly conserved catalytic and substrate-recognition motifs, allowing the use of specific FMK-linked oligopeptide inhibitors that bind to the active site of the protease and irreversibly inhibit caspase activity through coupling to FMK. In this series of experiments, SCL-1, SCC13, and HaCaT cells were cultured in normal culture medium or normal culture medium supplemented with imiquimod. Parallel cell cultures of imiquimod-treated cells were supplemented with the VAD peptide, which inhibits a common functional motif of all known caspases, or with oligopeptides specifically inhibiting caspases-1–4, -6, -8–10, and 13.

Inhibition of caspase-3, -8, -9, or -10 by specific oligopeptides resulted in statistically significant reductions in the pro-apoptotic effect of imiquimod similar to that seen with the VAD peptide (VAD, 60.1% reduction, 95% CI = 49.6% to 70.6%, P = .001; caspase-3, 56.6% reduction, 95% CI = 51.1% to 62.1%, P = .001; caspase-8, 67.8% reduction, 95% CI = 66.0% to 69.6%, P < .001; caspase-9, 64.8% reduction, 95% CI = 64.2% to 65.4%, P <.001; caspase-10, 68.9% reduction, 95% CI = 63.1% to 74.7%, P = .002, compared with imiquimod-treated cells; Fig. 3, B). Similarly, specific inhibition of caspase-4 and -6 resulted in statistically significant, albeit not complete, reduction of imiquimod-induced apoptosis (caspase-4,
39.9% reduction, 95% CI = 38.5% to 41.3%, \( P = .036 \) and caspase-6, 39.9% reduction, 95% CI = 39.6% to 40.2%, \( P = .003 \), compared with imiquimod-treated cells; Fig. 3, B). In contrast, when caspase-1, -2, and -13 were inhibited by specific oligopeptides, no statistically significant effect was observed on imiquimod-induced apoptosis (caspase-1, \( P = .46 \); caspase-2, \( P = .31 \); and caspase-13, \( P = .43 \), all compared with imiquimod-treated cells, Fig. 3, B). The lack of effect of specific oligopeptide inhibitors on caspase-2 is consistent with results showing that TNF-receptor signaling, which is transmitted in part via caspase-2, did not induce apoptosis in the HaCaT, SCL-1, and SCC13 cell lines studied here (49,52).

Imiquimod-Induced Apoptosis and Mitochondrial Cytochrome \( c \)

On exposure of cells to pro-apoptotic stimuli, cytochrome \( c \) is released from the mitochondria, enters the cytosol, and binds to APAF-1 and procaspase-9, which results in activation of caspase-9 and, eventually, activation of caspase-3 (49). Hence, to assess whether imiquimod may influence the release of mitochondrial cytochrome \( c \), we incubated HaCaT and SCL-1 cells with imiquimod or resiquimod and assessed cytochrome \( c \) within the mitochondrial and cytosolic compartments. After incubation with imiquimod, but not resiquimod, mitochondrial cytochrome \( c \) translocated to the cytosol (Fig. 4, A). We next assessed the kinetics of imiquimod-induced apoptosis and intracellular events (i.e., cytochrome \( c \) release and activation of caspase-3). We found that induction of apoptosis (Fig. 4, B) had similar kinetics to release of mitochondrial cytochrome \( c \) and activation of caspase-3—that is, release of cytochrome \( c \), activation of caspase-3, and maximum levels of DNA fragmentation were all detected after 24 hours (Fig. 4, C). To determine whether the different effects of imiquimod and resiquimod on the release of mitochondrial cytochrome \( c \) and

![Fig. 4. Imiquimod and Bel-2–dependent translocation of mitochondrial cytochrome \( c \). A) HaCaT cells were stably transfected with the pRES expression vector (four left lanes) or murine Bel-2 (four right lanes), and cultures of both transfected cell lines were incubated in normal culture medium (control) or in medium containing the Fas-activating monoclonal antibody (MAb) CH-11, imiquimod, or resiquimod for 24 hours. Mitochondria were purified and lysed in 1% Triton X-100, and cytochrome \( c \) expression was assessed by western blot analysis. B) HaCaT cells and SCL-1 cells were incubated in normal culture medium (control, filled bar) or in medium containing imiquimod (hatched bars) for 4, 12, 24, or 48 hours. Apoptosis was determined by assessing histone-bound DNA fragments. Error bars are shown as 95% confidence intervals. \( P \) values for the difference in apoptosis (absorbance value) relative to control are as indicated. n.s. = no statistically significant difference between the comparison groups. C) HaCaT cells were incubated in normal culture medium (control) or in medium containing imiquimod for 4, 12, or 24 hours. Release of mitochondrial cytochrome \( c \) (top panel), activation of pro-caspase-3 (middle panel), and activation of caspase-3 (bottom panel) were assessed by western blot analysis. The experiment shown is representative of three independent experiments showing similar results. D) HaCaT cells were incubated in normal culture medium (control; three left lanes) or in normal culture medium containing resiquimod (middle three lanes) or imiquimod (right three lanes) for 24 hours. Cells were lysed in hypotonic buffer, and the cytosolic (lanes 2; arrows) and mitochondrial (lanes 3) fractions were isolated. After lysis, an aliquot of each culture medium (lane 1) was extracted and separated by high-performance thin-layer chromatography. E) Mock-transfected HaCaT cells (HaCaT/pRES, two upper bars) and HaCaT cells overexpressing Bel-2 (HaCaT/bel-2, two lower bars) were incubated in normal culture medium (control) or in medium containing imiquimod. Apoptosis was assessed by determining histone-bound DNA fragments after 24 hours of incubation. Data are shown as the means and 95% confidence intervals (relative to the controls) of three independent cultures. *\( P < .001 \), compared with controls.
apoptosis of tumor cells are due to differences in the subcellular distribution of these two compounds, we assessed the presence of imiquimod and resiquimod in the cytosolic and membrane fractions of HaCaT cells incubated with either imiquimod or resiquimod. Although the solubility of both compounds in organic solvents suggests that they could be present within membranes, neither was definitively detected within the membrane fraction because a prominent background signal migrated at a similar position (Fig. 4, D). It is interesting, however, that whereas imiquimod was readily detected within the cytosolic fraction, little, if any, resiquimod was detected there (Fig. 4, D).

Thus, the different subcellular distribution of imiquimod and resiquimod may account, at least in part, for their differential effects on both release of mitochondrial cytochrome $c$ and induction of apoptosis in tumor cells.

To determine whether the imiquimod-induced release of cytochrome $c$ was by a direct or an indirect process, purified mitochondria from HaCaT cells were exposed to vehicle (PBS), imiquimod, or resiquimod for different time periods. Based on western blot analysis, a similar time-dependent release of cytochrome $c$ was found in isolated mitochondria incubated with imiquimod, resiquimod, and vehicle (data not shown). These results suggest that there was no direct release of cytochrome $c$ as a result of imiquimod treatment and that imiquimod-induced release of cytochrome $c$ in intact cells is mediated through proapoptotic proteins within the cell such as members of the Bcl-2 family.

**Imiquimod-Induced Apoptosis and Bcl-2**

The release of cytochrome $c$ is controlled by the relative amounts and associations of anti-apoptotic (e.g., Bcl-2, Bcl-xL) and pro-apoptotic (e.g., Bax, Bak, Bid) proteins in the outer membrane of the mitochondria (48). Because inhibition of imiquimod-stimulated activation of caspase-9 resulted in reduction of apoptosis in HaCaT, SCC13, and SCL-1 cells (Fig. 3, B), we hypothesized that imiquimod may have affected the mitochondrial Bcl-2-dependent apoptotic pathway. To test this hypothesis, HaCaT cells were transfected with a murine bcl-2 construct or the pIRES vector (42).

In the first series of experiments, mock transfectants (HaCaT/pIRES) and Bcl-2 overexpressing cells (HaCaT/Bcl-2) were incubated with normal culture medium or normal culture medium supplemented with imiquimod, resiquimod, or the CD95-stimulating CH-11 MAb. Western blot analysis revealed that cytochrome $c$ was strongly expressed in the mitochondrial fraction but not in the cytosolic fraction of HaCaT/pIRES cells after incubation with normal medium or resiquimod. However, after incubation with imiquimod or the CH-11 MAb, mitochondrial expression of cytochrome $c$ was reduced by 85%, with concomitant cytosolic translocation (Fig. 4, A). When HaCaT/Bcl-2 cells were incubated with imiquimod in an identical fashion, no translocation of cytochrome $c$ was detected (Fig. 4, A), suggesting that the effect of imiquimod on the release of cytochrome $c$ is Bcl-2-dependent.

To determine the relevance of these findings to apoptosis, we assessed the effect of Bcl-2 overexpression on imiquimod-driven DNA fragmentation. HaCaT/pIRES and HaCaT/Bcl-2 transfectant cells were incubated with normal culture medium or normal culture medium supplemented with imiquimod, resiquimod, or CH-11 MAb for 24 hours, and apoptosis was determined. As expected, both imiquimod and CH-11 MAb induced a marked increase in apoptosis in HaCaT/pIRES cells of 283.8% (95% CI = 273.8% to 293.8%) (Fig. 4, E; $p<.001$ compared with control-treated HaCaT/pIRES cells), whereas resiquimod did not induce an increase in apoptosis (data not shown). However, imiquimod did not induce apoptosis in HaCaT cells overexpressing Bcl-2 (Fig. 4, E), suggesting that imiquimod-induced apoptosis is indeed dependent on the Bcl-2 status of the cells.

**Imiquimod-Induced Apoptosis In Vivo**

To examine whether apoptosis is induced during clinical use of imiquimod, tumors from three patients with multiple superficial BCCs were treated with topical application of imiquimod (i.e., Aldara 5% cream; Fig. 5, A). Biopsies were taken from an untreated BCC and from a similar imiquimod-treated BCC after 4 days of topical application. Histochemical analyses revealed that a dense lymphocytic infiltrate surrounded the tumor tissue at this time, indicating an inflammatory response to imiquimod. Using a modified TUNEL assay, we subjected paraffin-embedded sections of these tumors to BrdU labeling of DNA fragments, and bound BrdU was detected by immunohistochemistry. Whereas few, if any, apoptotic cells were detected in the untreated BCCs, the number of apoptotic (i.e., BrdU-positive) cells in the treated BCCs greatly increased after 4 days of treatment with imiquimod (Fig. 5, B). Abundant apoptotic cells were detected within the tumor nests but not within the inflammatory infiltrate surrounding the tumors (Fig. 5, B). Similar induction of apoptosis in the tumor tissue, but not in the surrounding inflammatory infiltrate, was observed in another BCC tumor that was treated with topical imiquimod for only 3 days (data not shown). These observations, together with those in the in vitro SCC cell lines, suggest that imiquimod exerts a pro-apoptotic effect both in vitro and in vivo.

**DISCUSSION**

Based on several reports (6,8,39,49,52,54) of apoptotic resistance in nonmelanoma skin cancer, we hypothesized that therapeutic regimens overcoming such mechanisms of apoptosis resistance may be of use for the treatment of BCC and SCC. Therefore, we evaluated the biologic activity of two members of the imidazooquinoline family, imiquimod and resiquimod, which have shown anti-BCC tumor activity in clinical trials. Imiquimod, but not resiquimod, was able to bypass various signal transduction pathways (in keratinocyte-derived tumor cell lines) to induce activation of caspase-3 downstream (or independently) of membrane-bound death receptor activation. In addition, imiquimod was also capable of activating mitochondrial release of cytochrome $c$ in a Bcl-2-dependent fashion. The net result of these functions is tumor cell–specific apoptosis (summarized in Fig. 6), an action that may be relevant for effectively bypassing mechanisms developed by malignant tumors to resist natural apoptotic stimuli. Our data provide the first evidence, to our knowledge, for direct antineoplastic activity of imiquimod.

Preferential induction of tumor cell–specific apoptosis by a topical compound represents an exciting and novel anticarcinogenic mechanism that may greatly benefit the management of patients with BCC and SCC. Of particular note is our finding that imiquimod-induced apoptosis in tumor cells could not be antagonized by functional blockade of various membrane-bound death receptors, including Fas/APO-1 (CD95), TRAIL, and TNF receptors, which suggests that imiquimod-
induced apoptosis is independent from apoptosis mediated by membrane-bound death receptors. This action does not rule out the possibility that cytokines such as TNF-α or cytotoxic T cells could participate in imiquimod-induced tumor destruc-

Fig. 5. Imiquimod-induced apoptosis and basal cell carcinoma (BCC) in vivo. A) A typical superficial BCC from an 81-year-old patient with multiple BCCs was treated with imiquimod (i.e., Aldara 5% cream) for 4 consecutive days (right panel); an untreated BCC from the same patient served as a control (left panel). B) Paraffin-embedded sections of punch biopsy specimens of an untreated BCC (left panels) and a BCC treated with imiquimod (right panels) from the patient in (A). Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay; low-power magnification in the upper right panel (×40), high-power magnification in the lower three right panels (×200). Bottom right panel depicts uninvolved epidermis from an area treated with imiquimod. Dashed line indicates the location of the dermoepidermal junction. Arrows indicate examples of apoptotic (i.e., TUNEL-positive) cells. Scale bar = 50 μm. Apoptotic cells are seen within the tumor treated with imiquimod. Similar results were obtained for two other patients.

Fig. 6. Synopsis of selected key molecular events involved in apoptotic cell death, and the current concept of the molecular action of imiquimod based on our results. Membrane-bound death receptors (e.g., CD95, tumor necrosis factor [TNF]–related apoptosis-inducing ligand [TRAIL] receptors, and TNF receptors) are largely unaffected by imiquimod, which induces tumor-selective apoptosis via translocation of cytochrome c from the mitochondria to the cytosol in a Bcl-2-dependent manner, resulting in activation of caspase-9 and caspase-3. This mechanism presumably bypasses potential mechanisms of resistance developed by some tumors to apoptotic stimuli.

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tases of internal tumors. Moreover, each tumor type will need to be tested directly for its sensitivity to imiquimod because unique molecular mechanisms of apoptosis evasion may exist.

It is interesting that, although imiquimod exhibited marked pro-apoptotic activity on several keratinocyte-derived tumor cell lines, its analog, resiquimod, showed little, if any, such activity. The mechanisms underlying this difference in the biologic activities of these two structurally related compounds have not yet been clarified. However, at least two hypotheses can be delineated from the results of this study. First, the presence of imiquimod, but not resiquimod, within the cytosolic fraction, which also contains members of the Bcl-2 protein family, may account, at least in part, for the differences observed in biologic activity. Second, given that both imiquimod and resiquimod exert similar effects on nuclear factor-κB–mediated cytokine induction (36–38), it is conceivable that different structural motifs are required for pro-apoptotic and pro-inflammatory activities, respectively. Furthermore, additional side chains on resiquimod that are not present on imiquimod may cause steric hindrance of binding to molecular motifs involved in apoptosis. Further molecular modifications may unravel minimal structural requirements for either of the pro-inflammatory and the pro-apoptotic functions.

It cannot be ruled out that imiquimod exerts its in vivo tumor killing effect via induction of pro-apoptotic (e.g., cytokines and T-cell granymes) or pro-oncotic factors (e.g., reactive oxygen species released by activated macrophages). Nevertheless, it is reasonable to expect that a direct pro-apoptotic activity may contribute to imiquimod’s antitumor activity, because the concentration of imiquimod in the marketed formulation (i.e., Aldara 5% cream) is approximately 1000-fold higher than the concentration shown in this study to cause apoptosis in vitro. Based on phase I clinical trials (55), in which 0.9% of a single 5-mg imiquimod dose applied topically to the forearm of volunteers was recovered from urine and feces, one can assume that the local concentration of imiquimod in the skin in vivo will be at least within the range shown to induce apoptosis in tumor cells in vitro.

The pro-apoptotic effect of imiquimod occurred independently of T-cell– or dendritic-cell–derived cytokines because dendritic cells, which are known to be the primary drug-responsive cell type and the principal source of released cytokines (24–28,30–32), and other leucocytes were not included in our cell cultures. The observation that resiquimod did not lead directly to apoptosis does not indicate how that compound might behave in a clinical setting where cytokines and T cells would be involved. However, the concentration of resiquimod currently under development is only 1.3 logs higher than the highest doses tested in vitro; thus, a direct apoptosis effect of resiquimod is unlikely to be present in vivo.

In summary, our results demonstrate that a topical compound may directly and selectively induce apoptosis in skin tumors. Such an approach would avoid the many potential side effects of systemic treatment or surgery. By inducing apoptosis specifically in the tumor cells, a synergistic enhancement of the immunomodulatory effects of imiquimod may speed the clearance of epithelial skin tumors.

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Notes

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