Oncogenic ras has been shown to downregulate Fas receptor expression and increase Fas ligand expression and thus contribute to resistance to Fas-mediated cell death in several cell types. The effects of ras on Fas-mediated apoptosis have not been studied in melanoma. We studied the effects of activated N-ras by measuring Fas, Fas ligand, and FLIP expression as well as susceptibility to Fas-ligand-induced cell death in transfectants of WM35, a radial growth phase human melanoma cell line. Based on quantitative polymerase chain reaction and fluorescence-activated cell sorter analysis, we found that the ras transfectants expressed less Fas mRNA and surface Fas receptor. Cr51 release cytotoxicity assays demonstrated less susceptibility to Fas-mediated apoptosis in ras transfectants, correlating with the Fas mRNA and protein expression results. Ras inhibition with the specific inhibitor FTI-277 showed that downregulation of Fas in the ras transfectants could be reversed. This correlates with cytotoxicity experiments showing that ras inhibition increases susceptibility to Fas-mediated apoptosis. The control transfectants expressed FLIP but ras did not affect FLIP expression. The control and ras transfectants did not express Fas ligand as demonstrated by reverse transcriptase polymerase chain reaction and fluorescence-activated cell sorter analysis. Cytotoxicity assays further confirmed that these melanoma ras transfectants do not express functional Fas ligand. These results suggest that ras contributes to tumor progression by decreasing susceptibility to Fas-mediated cell death at least in part through downregulation of Fas receptor at the transcriptional level. Key words: FLIP/Fas ligand/FT1-277. J Invest Dermatol 119:556–561, 2002

Regulation of Fas-Mediated Apoptosis by N-ras in Melanoma

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Oncogenic ras mutations have been well associated with tumor progression. Approximately 36% of melanomas have a ras mutation, of which N-ras is the most common (Ball et al, 1994). Ras mutations are more commonly associated with melanoma progression from radial growth phase to vertical growth phase. Melanoma demonstrates increased cell motility and loss of anchorage-dependent growth when mutated ras is introduced, thus increasing its tumorigenicity (Fujita et al, 1999).

The ras gene product is synthesized as a cytoplasmic precursor, which requires post-translational processing for activity. The critical modification is farnesylation of the cysteine residue in the CaaX motif (where C = cysteine, a = aliphatic, X = any amino acid) located near the carboxyl terminus of all ras proteins. Once farnesylated, the mature ras protein is attached to the plasma membrane where it participates in signal transduction pathways that control cell growth and differentiation.

Ras contributes to tumor progression by altering normal growth and proliferation pathways. Ras affects susceptibility to apoptosis through changes in normal apoptotic machinery (Evan and Littlewood, 1998; Lowe and Lin, 2000). Ras has been shown to protect various cell types from anoikis (Khawaja et al, 1997), c-myc-induced apoptosis (Kauffmann-Zeh et al, 1997), and death receptor pathways such as Fas-mediated apoptosis (Fenton et al, 1998; Peli et al, 1999; Yang et al, 2000).

Fas-mediated apoptosis involves many factors to induce cell death. The Fas receptor (CD95, APO-1) is a type I transmembrane receptor that is a member of the tumor necrosis factor (TNF) receptor family (Nagata, 1997). It has a cytoplasmic death domain that binds with Fas-associated death domain (FADD/MORT1) upon activation by Fas ligand (FasL) (Boland et al, 1995; Chinnaiyan et al, 1995). Caspase-8 (FLICE/MACH) is activated by binding to FADD, which is already bound to the Fas receptor to form the death-inducing signaling complex (DISC). Caspase-8 activates downstream effector caspases, which leads to cell death.

FasL is a type II transmembrane receptor that is a member of the TNF family (Suda et al, 1993; Suda and Nagata, 1994). It is expressed on activated T cells and binds to Fas receptor to induce cell death in Fas-bearing target cells. FasL expression in immune-privileged sites such as the eye and testis (Bellgrau et al, 1995; Griffith et al, 1995) induces cell death of Fas-bearing activated T cells and reduces inflammation. Tumors exhibit this type of defense against activated T cells by expressing FasL (Walker et al, 1997).

Flice inhibitory protein (FLIP, I-FLICE, CASPER) is a downstream inhibitor of Fas-mediated apoptosis. It binds to FADD in the DISC and prevents activation of caspase-8 (Hu et al, 1997; Irmler et al, 1997). Cells that exhibit high levels of FLIP show resistance to Fas-mediated cell death (Hu et al, 1997; Irmler et al, 1997).
Many studies have investigated the different factors involved with Fas-mediated cell death in melanoma. Fas receptor expression is variable depending on the cell line or tumor stage (Ugurel et al., 1999). Conflicting data exist on whether or not melanoma expresses FasL. Some studies have found FasL to be present (Hahne et al., 1996; Ugurel et al., 1999) whereas other studies have found that FasL is not expressed in melanoma (Chapcelli et al., 1999). Melanoma cell lines express FLIP but the levels are variable depending on the cell line (Irmler et al., 1997; Ugurel et al., 1999). The effects of ras on Fas receptor expression, FasL expression, FLIP expression, and susceptibility to FasL-induced cell death, however, have not been studied in melanoma. We report that ras decreases susceptibility to Fas-mediated cell death through downregulation of Fas receptor expression with no effects on FLIP or FasL expression.

MATERIALS AND METHODS

Cell lines and chemicals WM35 radial growth phase human melanoma cell line was obtained from the Wistar Institute (Philadelphia, PA). Plasmid control transfecteds (WM35 neo) and N-ras transfecteds (WM35 N-ras) of WM35 were established as described in a previous study (Fujita et al., 1999). They were grown in 5% fetal bovine serum RPMI 1640 (Gibco BRL), 400 μg per ml G418 (Gemini Bioproducts, Woodland, CA). Jurkat and K562 cell lines were purchased from ATCC (Rockville, MD). K562 cells were used as negative controls as they do not express Fas or FasL. K562 cells were transfected with human FasL to establish G10 transfectants as described in a previous study (Duke et al., 1999). G10 cells were used as a positive control for FasL in immunofluorescent staining and as effector cells in cytotoxicity assays. L1210-Fas is a mouse leukemia cell line transfected with human Fas. L1210-Fas cells were used as positive control target cells in cytotoxicity assays as they express Fas. L1210-Fas cell line was a gift from Dr. P. Golstein (Marseille, France). K562 cells were grown in suspension culture medium (RPMI 1640 containing 10% heat inactivated fetal bovine serum, 2 mM glutamine, 42 μM 2-mercaptoethanol, 100 μg per ml gentamicin). G10 and L1210-Fas cells were grown in the same suspension culture medium with 600 μg per ml G418. The ras farnselylation inhibitor FTI-277 was purchased from CalBioChem (La Jolla, CA).

Antibodies Phycocerythrin-labeled anti-human CD95, phycocerythrin-labeled mouse IgG control, biotinylated anti-human CD95L, and streptavidin—phycoerythrin antibodies were purchased from Pharmingen (San Diego, CA). Immunoblot antibody anti-human FLIP (rabbit polyclonal IgG) was obtained from Upstate Technology (Lake Placid, NY). Goat antirabbit horseradish peroxidase (HRP) antibody was purchased from Biorad (Hercules, CA). Anti-actin (goat polyclonal IgG) and antigoat HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Ras activation assays Ras activation assays were performed using the manufacturer’s reagents and instructions (Upstate Biotechnology, Lake Placid, NY). Briefly, cells were harvested and lysed using the manufacturer’s lysis buffer. Raf-1 RBD agarose conjugated beads were used to precipitate activated ras proteins. Proteins were detached from beads and analyzed by Western blot.

Immunofluorescent staining Cells (1 x 10^6) were harvested using phosphate-buffered saline/ethylenediamine tetracetic acid (PBS/EDTA). Cells were centrifuged at 1000 rpm (Beckman TJ-6 centrifuge) for 5 min at room temperature. Cells were washed in PBS before they were resuspended in 100 μl antibody staining solution (0.1% sodium azide, 5% goat serum in PBS). Ten microliters of antihuman CD95 or anti-human CD95L antibody were added to each sample and the samples were incubated at room temperature for 15 min in the dark. Samples were washed with PBS twice before 10 μl streptavidin—phycoerythrin antibody was added to samples stained with anti-CD95L. Samples were incubated at room temperature for 10 min in the dark, were washed with PBS twice, and then were fixed with 1% paraformaldehyde in PBS. Cells were analyzed by flow cytometry (Beckman Coulter XL, Beckman Coulter, Miami, FL) at the University of Colorado Health Sciences Center Cancer Center Flow Cytometry Core (Denver, CO).

Cytotoxicity assays Cells were harvested with PBS/EDTA. Sodium chromate (100 μCi, ICN Pharmaceuticals, Irvine, CA) was added to 5 x 10^5 cells in 1 ml medium (RPMI 1640 with 10% fetal bovine serum) and incubated at 37°C in 5% CO2 humidified air for 1 h. Cells were washed with 2 ml medium twice and incubated at 37°C in 5% CO2 humidified air for 1 h in 2 ml medium. Cells were washed with 2 ml medium, centrifuged, and resuspended to a concentration of 5 x 10^5 cells per ml. Aliquots of 100 μl of labeled cells (5000 cells) were loaded into 96-well V-bottomed microtiter plates in triplicate. Triton X (1%) was added to determine maximum 51^Cr release. Medium was added to determine spontaneous release. Effectors were diluted to appropriate concentrations for various effector to target ratios. Samples were centrifuged (1000 rpm and the centrifuge was immediately turned off with brake off) and incubated 12-14 h at 37°C in 5% CO2 humidified air. Following incubation, 100 μl of cell-free supernatant was harvested and read in a gamma counter (Beckman Gamma 5500, Beckman Coulter, Fullerton, CA). Percent specific cell death was calculated using the following formula: (c - s)/(m - s) X 100, where c represents the amount of radioactivity released from target cells incubated with effector cells, s represents the amount of spontaneous release of radioactivity from the target cells (target cells incubated with medium), and m represents the maximum amount of radioactivity released when target cells are lysed with Triton X-100.
Real-time polymerase chain reaction (PCR) RNA extracts were prepared according to the Trizol Reagent protocol (Sigma, St. Louis, MO). Primers forward 5'-ACCCGCTCAGTACGGAGTTG-3' and reverse 5'-GGTAGGAGGGTCCAGATGCC-3' and TaqMan probe 5'-TCCGAGGATTGCTCAACACCATTC-3' were used for real-time PCR. The final concentration of RNA samples was 1 µg per µl in sterile diethyl pyrocarbonate treated water. Samples were given to the Real Time PCR Core Facility at the University of Colorado Health Sciences Center (Denver, CO). Real-time PCR was carried out using ABI PRISM 7700 following the manufacturer’s instructions (PE Biosystems, CA). The ABI PRISM 7700 determines the initial copy number of the target template by analyzing the cycle-to-cycle change in fluorescence signal as a result of the amplification of template during PCR. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number. The initial template concentration from each sample is determined by comparison with the standard curve.

Reverse transcriptase PCR RNA extracts were prepared according to the Trizol Reagent protocol (Sigma). Approximately 5 µg of RNA was converted to cDNA using Superscript II-RT (Gibco BRL) according to the manufacturer’s instructions. FasL DNA product was amplified using 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 mM 5’ and 3’ oligonucleotide primers, and 2.5 U Taq polymerase (Gibco BRL) in a final volume of 50 µl. The PCR took place in 0.2 ml Gene Amp microtubes (Perkin Elmer, Cetus, Norwalk, CT) and samples of DNA were run in the automated Cycler GeneAmp PCR System 9600 (Perkin Elmer, Cetus) using the following settings: 94°C for 1 min, 55°C for 1 min, 72°C for 30 s for 30 cycles. PCR products were run through 1% agarose gels with 0.5 µg per ml ethidium bromide. Gels were analyzed on Chemi-doc (Biorad, Hercules, CA). Primer sequences for FasL were forward 5'-ATATTCTCAAATGAGTACATCAGT-3' and reverse 5'-CACTGAATACAACATTCTCGG-3'. Primer sequences for glyceraldehyde-3-phosphate dehydrogenase were 5’-GGTCGGAGTCAACGGATTTG-3’ and 5’-ATGACC-CCAGCCCTTCTCCAT-3’.

Western blots Cells were detached with PBS/EDTA. Samples were washed in PBS and lysed in 100 µl protein lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS, 1 mM EDTA, 0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor (Boehringer Mannheim, Mannheim, Germany)]. Samples were placed on ice and analyzed for protein concentration using the BioRad Protein Assay. Protein concentration was determined by analysis on Bio-Kinetics reader EL312e (Biotek Instruments, Winooski, VT) at wavelength 570 nm. In each lane, 100 µg of protein was loaded into polyacrylamide gels (4% stacking and 10% resolving gels). Gels were run at 12 mA current per gel. Proteins were transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA) on Biorad Transblot SD Semi-Dry Transfer Cell apparatus at 25 V for 15 min. Membranes were probed with the antibody of interest in PBS with 5% milk and 0.02% sodium azide. Blots were washed with PBS for 5 min, then TBS/Tween for 5 min x 3, then PBS for 5 min. They were probed with HRP-conjugated secondary antibody in PBS with 5% milk, and were incubated in HRP substrate (Pierce West Pico or Pierce West Fempto developing solutions, Rockford, IL) for 5 min at room temperature. They were viewed and analyzed using Chemi-doc (Biorad).

RESULTS Effects of N-ras on susceptibility to Fas-mediated apoptosis To determine relative amounts of ras activity in WM35 transfectants, we tested control (WM35 neo) and ras

Figure 2. N-ras decreases surface Fas receptor expression in WM35 cells. (A) WM35 neo and WM35 N-ras clones were stained with immunofluorescent antibodies and analyzed by FACS. (B) Immunofluorescence ratios determined by dividing mean fluorescence of cells stained with antihuman Fas by mean fluorescence of cells stained with mouse IgG (control antibody). The graph represents results of three independent experiments. SEM is shown (n = 3), p-value < 0.05 for all N-ras clones. (C) WM35 N-ras S1 clones treated with DMSO (Nras DMSO) or FTI-277 10 µM (Nras FTI-277) for 48 h were stained for surface Fas expression. Final DMSO concentration for both conditions was 0.1%. This graph represents results from four independent experiments. SE is shown (n = 4), p-value < 0.005.
(WM35 N-ras) transfectants in ras activity assays. WM35 N-ras had 30% more ras activity than WM35 neo (data not shown). We then tested WM35 neo and WM35 N-ras transfectants in chromium release cytotoxicity assays to determine the effects of N-ras on susceptibility to Fas-mediated apoptosis. Both WM35 neo and WM35 N-ras clones were more resistant to Fas-mediated cell death than the positive control cell line (Fig 1A). All three WM35 N-ras clones showed less susceptibility to Fas-mediated apoptosis than WM35 neo, however (Fig 1A). Fas-mediated cell death of WM35 neo was comparable to that of the parent cell line WM35 (data not shown).

Inhibition of ras farnesylation is an effective way to block ras activity (Gibbs et al, 1993; Lerner et al, 1995). Farnesylation inhibitors may selectively prevent development of tumors with deregulated ras signals by blocking the function of mutant ras in transformed (neoplastic) cells without affecting normal cells (Kohl et al, 1994, 1995). Treatment of WM35 N-ras with FTI-277, a farnesylation inhibitor, decreased ras activity by 20% (data not shown). When WM35 N-ras was treated with FTI-277, susceptibility to Fas-mediated cell death was significantly increased compared to untreated WM35 N-ras cells (Fig 1B). Treatment of WM35 neo cells with FTI-277 did not affect susceptibility to Fas-mediated cell death (data not shown).

**Effects of N-ras on surface Fas receptor expression** To further understand the mechanism by which ras affects susceptibility of these melanoma cells to Fas-mediated apoptosis, we investigated the effects of ras on Fas surface receptor expression. WM35 neo and WM35 N-ras clones were stained with immunofluorescent antibodies for Fas and analyzed by fluorescence-activated cell sorter (FACS). Immunofluorescence ratios showed that WM35 N-ras clones expressed 1.5–2-fold less surface Fas receptor than WM35 neo (Fig 2A, B). The difference in background fluorescence of WM35 neo and WM35 N-ras is probably due to the larger morphologic size of ras transfectants. Treatment of WM35 N-ras with FTI-277 increased the surface expression of the Fas receptor 1.2-fold (Fig 2C), correlating with the increase in cell death as seen in (Fig 1B). FTI-277 did not change Fas surface receptor expression in WM35 neo cells (data not shown).

**Effects of N-ras on Fas mRNA expression** To determine if activated N-ras affected Fas expression by transcriptional regulation, we used real-time PCR to quantitate Fas mRNA levels in these cell lines. WM35 N-ras showed a 44% decrease in Fas expression at the mRNA level (Fig 3A). Inhibition of ras with FTI-277 showed a 157% increase in Fas mRNA levels in WM35 N-ras (Fig 3B) correlating with the trend of increased surface protein expression and increased susceptibility to Fas-mediated cell death.

**Effects of N-ras on FLIP expression** Factors downstream of Fas receptor activation such as FLIP may also influence the susceptibility of a cell to Fas-mediated apoptosis. FLIP protein levels were measured to determine if ras regulated Fas-mediated apoptosis by modulating expression of this protein. Western analysis showed that there was no difference in FLIP protein expression between WM35 neo and WM35 N-ras (Fig 4A). Ras inhibition also showed no effects on FLIP protein expression (Fig 4B).

**Effects of N-ras on FasL expression** Various studies have shown conflicting results about FasL expression in melanoma. We tested for FasL expression by staining for surface protein and by reverse transcriptase PCR. WM35 neo did not express FasL and expression of activated ras in WM35 N-ras did not induce FasL expression (Fig 5A, B). Furthermore, when WM35 neo and WM35 N-ras were used as killer cells in cytotoxicity experiments, they did not induce cell death in susceptible cells (Fig 5C).

**DISCUSSION**

The mechanism by which melanoma progresses from radial growth phase to the more invasive vertical growth phase may be related to decreased susceptibility to triggers of cell death. Activated ras has been associated with tumor progression in many cancers and may contribute to tumor progression in melanoma. Our studies support that activated ras affects Fas-mediated cell death by downregulating Fas expression at the transcriptional level and thus decreasing the susceptibility of these melanoma cells to Fas-mediated apoptosis. These results are consistent with findings from other studies, which demonstrate transcriptional regulation of Fas receptor expression by ras in other cell types (Fenton et al, 1998; Peli et al, 1999). The promoter region of the Fas receptor gene is CpG dinucleotide rich, which suggests that gene regulation may be affected by methylation (Behrmann et al, 1994). Others have shown that blocking methylation in ras-transfected epithelial cells leads to increased Fas receptor expression, further supporting the effects of ras on transcriptional regulation of this death receptor (Peli et al, 1999).

The increase in Fas mRNA expression was much greater, however, than the modest increase in surface Fas receptor expression when these cells were treated with ras inhibitors. This
may represent the increase in transcriptional activity that has not yet been manifested at the protein level. It is possible that these inhibitors may have an effect on translational regulation and translocation of the Fas receptor to the cell membrane surface, which are not well understood.

Fas receptor expression, however, does not necessarily correlate with susceptibility to FasL-induced cell death (Owen-Schaub et al., 1994; Shima et al., 1995). Factors downstream of Fas receptor activation such as FLIP, IAPs, and activation of bcl-2 family members may influence a cell's susceptibility to Fas-mediated apoptosis regardless of the surface Fas receptor expression. FLIP expression has been described as a mechanism by which tumors are able to escape immune surveillance (Djerbi et al., 1999; French and Tschopp, 1999; Medema et al., 1999). Our studies show that FLIP is present in melanoma, which is consistent with other studies. Activated ras, however, does not affect FLIP expression in these cells.
cells and thus is not a mechanism by which ras protects this melanoma cell line from Fas-mediated apoptosis. Other studies have suggested susceptibility to Fas-mediated apoptosis may involve factors from the caspase-9 pathway such as bcl-2 and bcl-xL (Li et al., 1998; Ugurel et al., 1999). We are currently investigating the effects of the bcl-2 family on susceptibility of these ras transfectants to Fas-mediated cell death.

Other tumors express FasL and may use this as a mechanism to evade the immune response. For this particular melanoma cell line, WM35, however, FasL is not expressed and ras does not induce FasL expression as seen in human glioma cells (Yang et al., 2000). Thus, FasL does not play a role in increasing the tumorigenicity of WM35 as it advances from radial growth phase to vertical growth phase.

Our studies suggest that ras may contribute to the tumor progression of melanoma by increasing resistance to Fas-mediated apoptosis by downregulating Fas receptor expression. More studies need to be conducted further understand the effects ras may have on factors downstream of Fas receptor expression. Ras inhibitors such as farnesylation inhibitors have demonstrated effects on tumor regression and prevention of tumor progression in mice (Sebti and Hamilton, 2000). The increase in susceptibility to Fas-mediated cell death in melanoma induced by ras inhibition suggests that farnesylation inhibitors may be efficacious in treatment of melanoma if used in combination with other immunotherapeutic or chemotherapeutic agents.

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