Selective Expression of FLIP in Malignant Melanocytic Skin Lesions

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FLIP (FLICE Inhibitory Protein) is a recently identified intracellular inhibitor of caspase-8 activation that potently inhibits cell death mediated by all death receptors including Fas and TRAIL. FLIP has recently been shown to favor tumor growth and immune escape in mouse tumor models. We analyzed FLIP expression by immunohistochemistry in a panel of 61 benign and malignant human melanocytic skin lesions. FLIP expression was undetectable in all but one benign melanocytic lesion (31/32, 97%). In contrast, FLIP was strongly expressed in most melanomas (24/29 = 83%). Overexpression of FLIP by transfection in a Fas- and TRAIL-sensitive human melanoma cell line rendered this cell line more resistant to death mediated by both TRAIL and FasL. Selective expression of FLIP by human melanomas may confer *in vivo* resistance to FasL and TRAIL, thus representing an additional mechanism by which melanoma cells escape immune destruction. ***Key words: apoptosis/death receptors/melanoma. J Invest Dermatol 117:360–364, 2001***

Apoptosis is a tightly regulated form of cell death that is implicated in the defence against pathogens and cancer. Apoptotic cell death can be triggered by various stimuli, including engagement and activation of death receptors belonging to the TNF receptor (TNFR) superfamily (TNFR-1, Fas/Apo1/CD95, DR3/Apo3, TRAIL-R1, TRAIL-R2, and DR6) (for review see Ashkenazi and Dixit, 1999). Death receptor mediated apoptosis has been shown to be crucial to the proper homeostasis and function of the immune system (Nagata, 1997; Ashkenazi and Dixit, 1999).

Fas ligand (FasL, CD95L), the prototype death ligand, has been shown to be one of the two major effector molecules of cytolytic T lymphocyte (CTL)-mediated cytotoxicity (Kägi *et al*, 1994a, b; Lowin *et al*, 1994a, b). CTL-mediated cytolysis involves transient expression of FasL on activated lymphocytes, and subsequent cytolysis of Fas-bearing target cells following Fas-FasL cross-linking. A functional Fas signaling pathway in tumor cells is therefore a prerequisite for effective Fas-mediated tumor cell destruction by the immune system. TRAIL, a more recently identified ligand for the death receptors TRAIL-R1 and R2, has been shown to quite selectively induce tumor cell death, and is expressed upon activation of T cells, natural killer cells, monocytes, and blood dendritic cells (Ashkenazi and Dixit, 1998; Thomas and Hersey, 1998; Zamai *et al*, 1998; Fanger *et al*, 1999; Griffith *et al*, 1999; Kayagaki *et al*, 1999). TRAIL has recently been found to mediate CD4 CTL-mediated killing of Jurkat T cells, autologous and allogeneic melanoma cells *in vitro*, including FasL-resistant melanoma cells (Kayagaki *et al*, 1999; Thomas and Hersey, 1998). Taken together evidence now exists suggesting that death receptor signaling mediated by FasL and TRAIL may be implicated in the cellular immune response to melanoma. Consequently, inhibition of these death receptor pathways may favor immune escape.

Recently, a new family of viral inhibitors of death receptor-mediated cell death, named viral FLIP (FADD-like IL1β-converting enzyme (FLICE)/caspase-8 inhibitory proteins) (vFLIP) has been identified, and shown to act as dominant negative inhibitors of FADD, a crucial mediator of death receptor signaling (Thome *et al*, 1997). A human cellular homolog of FLIP (also called CASPER, iFLICE, FLAME-1, CASH, CLARP, MRIT, and Usurpin) was subsequently identified, and shown to exist in two protein forms: a long form ($FLIP_L$) containing two death effector domains (DED) and an inactive caspase domain, and a short form ($FLIP_S$), containing only two DED (Irmler *et al*, 1997). Overexpression of FLIP has been shown to potently inhibit death receptor-mediated apoptosis by binding to FADD and caspase-8 and thus specifically inhibit apoptosis mediated by all currently known death receptors (Goltsev *et al*, 1997; Hu *et al*, 1997; Irmler *et al*, 1997; Rasper *et al*, 1998). FLIP is detectable in melanoma cell lines *in vitro* (Irmler *et al*, 1997), and has been shown in animal tumor models to favor escape from T cell immunity despite a functional perforin-granzyme cytolytic pathway (Djerbi *et al*, 1999; Medema *et al*, 1999). Because little is known about FLIP expression with respect to human tumorigenesis *in vivo*, we have immunohistochemically analyzed FLIP expression in biopsies of cutaneous melanocytic lesions with respect to tumor progression and assessed its effect on TRAIL sensitivity in FLIP-transfected melanoma cell lines.

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Abbreviations: CTL, Cytotoxic T lymphocyte; DED, Death effector domain; FADD, Fas-associated death domain protein; FLICE, FADD-like ICE (caspase-8); $FLIP_L$, Long FLICE inhibitory protein; $FLIP_S$, Short FLICE inhibitory protein; c-FLIP, Cellular FLIP; v-FLIP, Viral FLIP; TRAIL, TNF-related apoptosis-inducing ligand.

MATERIALS AND METHODS

Tissue samples Sixty-one paraffin-embedded cutaneous melanocytic lesions were used for immunohistochemical analysis. The histologic diagnoses were as follows: 23 common nevi, nine atypical nevi, eight superficial spreading melanomas (mean Breslow index 0.66 mm, range 0.25–1.25), 10 nodular melanomas (mean Breslow index 5.17 mm, range 1.66–10.5), 11 cutaneous metastatic melanomas.

Antibodies and recombinant proteins Two polyclonal antibodies (AL129 and AL148) directed against distinct recombinant fragments of human FLIP were generated in rabbits (Eurogentec, Seraing, Belgium) and controlled for specificity by immunoblotting of protein extracts from human FLIP- or mock-transfected Jurkat cells.

Recombinant soluble Flag-tagged TRAIL and FasL (Alexis, San Diego, CA) were used at 100 ng per ml and mixed with enhancer (Alexis) at 1 μg per ml to increase the activity of TRAIL and FasL through aggregation.

Immunohistochemistry Paraffin-embedded (6 μm) tissue sections mounted on silane-treated slides were deparaffinized in xylene, rehydrated, and processed for antigen retrieval by microwave heating (710 W) for 10 min in citrate buffer (10 mM, pH 6). Endogenous peroxidase activity was quenched with 0.03% H_2O_2 and nonspecific binding prevented by preincubation in PBS-1% BSA. For FLIP analysis, the polyclonal anti-FLIP antibodies AL129 and AL148 were used at a concentration of 25 μg per ml in PBS-1% BSA-0.05% Tween. Isotype-matched rabbit IgG was used at the same concentration as a negative control. Antibody-coated slides were incubated in a humid chamber for 2 h, washed twice in PBS, incubated for 40 min with biotin-conjugated antirabbit-IgG diluted (1:250 in PBS-1% BSA-0.05% Tween), followed by 30 min streptavidin-peroxidase (StreptABComplex/HRP, Dako, Glostrup, DK). After another wash, 3-amino-9-ethylcarbazole (AEC) and H_2O_2 were added for 10 min, and then slides were washed and counterstained with hematoxylin.

Semiquantitative analysis of intensity of FLIP immunostaining intensity was performed by rating staining intensity as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. In all experiments similar results were obtained using both antihuman FLIP antibodies (AL129 and AL148).

Morphometric analysis of the percentage of melanocytic cells expressing FLIP was performed by counting a minimum of 200 melanocytes per immunostained section.

Immunoblotting Whole cell lysates (50 μg) were resolved in 10% SDS-PAGE, transferred to nitrocellulose, and blotted with polyclonal antihuman FLIP antibody (AL129 or AL148, 1 μg per ml) for 1 h, then incubated for 45 min with HRP-conjugated antirabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) at a 1:2000 dilution. Signals were detected using the ECL system (Amersham, Arlington Heights, IL).

Cell culture and FLIP transfection The human melanoma cell line WK, derived from a primary cutaneous melanoma of superficial speading type was cultured on plastic in minimal essential media (MEM) with Earle's balanced salt solution supplemented with 10% fetal calf serum and maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air (Byers *et al*, 1991). Stable geneticin-resistant transfectants of WK cells were generated by electroporation of 5×10^6 cells in 800 μl PBS, mixed with pCR-3 (Invitrogen) plasmid with or without the human FLIP insert, at 250 V and 960 μF. Starting 48 h after transfection, geneticin was added at 400 μg per ml until growth of resistant clones. Resistant clones were isolated, amplified, and analyzed for FLIP gene expression using reverse transcriptase-polymerase chain reaction (RT-PCR).

Extraction of RNA and RT-PCR Total RNA was prepared from cells using TRIzol Reagent (Gibco BRL, Life Technology, Basel, Switzerland). Briefly, cells were lyzed by adding 1 ml of TRIzol Reagent, RNA was precipitated from the aqueous phase and resuspended in water.

Total mRNA (500 ng) was reverse transcribed and amplified using a one-step RT-PCR kit (Acees RT-PCR System, Promega, Madison, WI). The nucleotide sequences of the sense and antisense primers for FLIP were 5′-ATTGGTGAGGATTTGGATAA-3′ and 5′-TGGGCG-TTTTCTTTCTTGTC-3′, respectively. For actin, the sense and antisense primers were 5′-TGATGGACTCCGGTGACGG-3′ and 5′-TGT-CACGCACGATTTCCCGC-3′, respectively. Amplification was carried out for 25 cycles using a step-cycle that consisted of denaturation [94°C; 1 min (for FLIP) or 94°C; 30 s (for actin)], annealing [55°C; 30 s (for FLIP) or 62°C; 1 min (for actin)] and extension [72°C; 1 min (for FLIP) or 68°C; 2 min (for actin)]. The final extension was allowed to proceed for 7 min. After PCR, 10% (i.e., 5 μl) of each reaction mixture were subjected to electrophoresis on a 1% agarose gel containing 0.5 μg per ml ethidium bromide in 1 × Tris-acetate electrophoresis buffer. The gel was photographed under ultraviolet light.

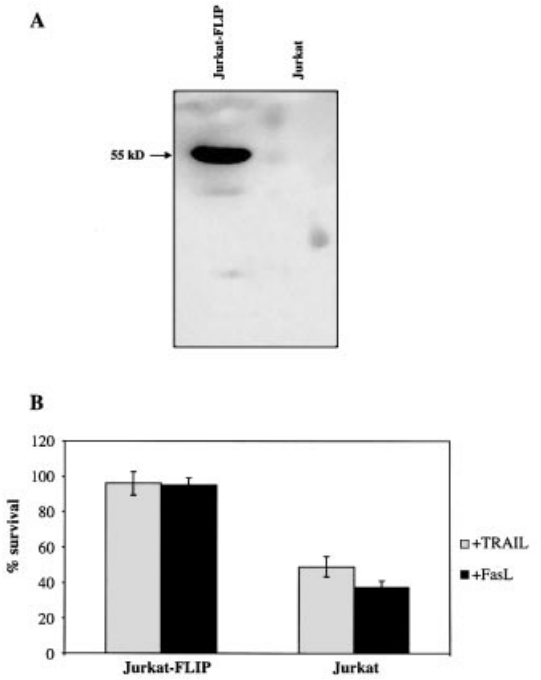

Figure 1. The AL129 antibody specifically recognizes FLIP protein. (*A*) Immunoblot analysis of lysates from FLIP- (left lane) and mock-transfected Jurkat cells using the rabbit antihuman FLIP antibody AL129. (*B*) Sensitivity of Jurkat and Jurkat-FLIP cells to recombinant FasL and TRAIL.

Measurement of TRAIL- and FasL-mediated cell death 10^4 cells resuspended in 50 μl were plated in 96 well microtiter plates and allowed to adhere. After adhesion, recombinant soluble TRAIL (or FasL) plus enhancer (Alexis) diluted in 50 μl culture medium were added in order to obtain a final TRAIL (or FasL) concentration of 100 ng per ml and an enhancer concentration of 1 μg per ml. Control wells received 50 μl of culture medium alone. Cell viability was assessed after 16 h using the WST-1 cell proliferation kit according to manufacturer's instructions (Roche Diagnostics, Rotkreuz, Switzerland). Briefly, 10 μl of WST-1 reagent was added to each well and incubated at 37°C for 45 min. Thereafter absorbance was measured at 450 nm. Cell survival after TRAIL (or FasL) addition was calculated with respect to control cells having received culture medium alone.

RESULTS

Specificity of anti-FLIP antibodies The specificity of the polyclonal Ab was tested by immunoblotting of protein extracts from Jurkat cells transfected with human FLIP. Both anti-FLIP Ab AL129 (**Fig 1*A***) and AL148 (not shown) selectively detected a band of the expected size (55 kDa) in the FLIP transfected cell line that was shown to have acquired resistance to FasL and TRAIL (**Fig 1*B***). We further tested the specificity of these antibodies using cells cultured *in vitro*. Immunohistochemical data obtained on cytospins of wild-type and FLIP-transfected Jurkat cells with the same antibodies used for the immunohistochemical study perfectly correlated with FLIP expression as quantified by RNase protection and western blot on the same cells (data not shown).

FLIP expression in benign melanocytic skin lesions FLIP expression was analyzed using both anti-FLIP polyclonal Ab in 23 common nevi and nine atypical nevi. As shown in **Figs 2** and **3**, FLIP expression was undetectable in 22 of 23 common nevi and all

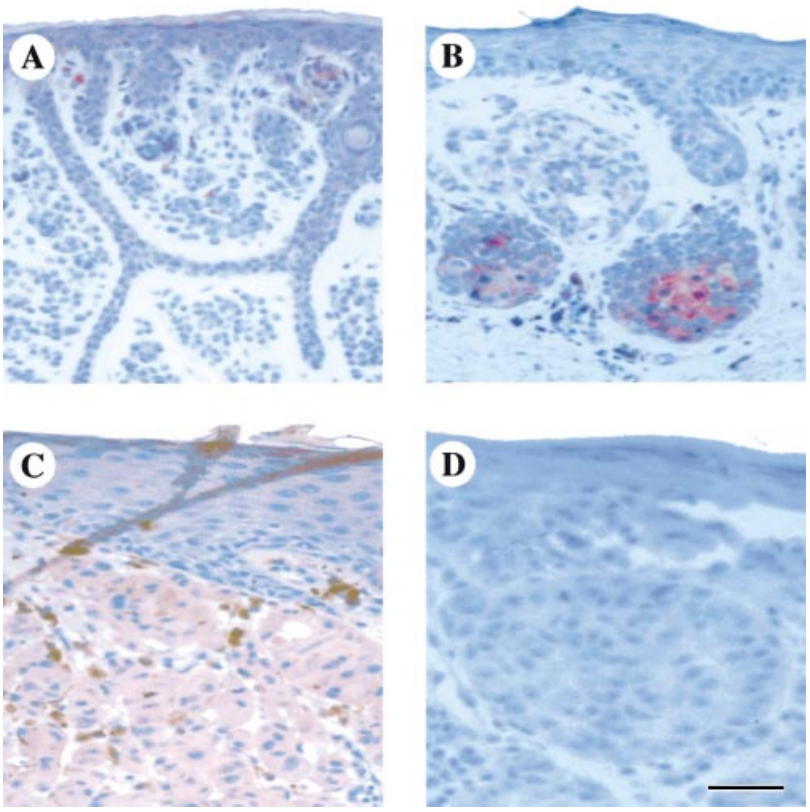

Figure 2. Selective expression of FLIP in melanomas. (*A–C*) Immunohistochemical staining with AL129 anti-FLIP antibody of an atypical nevus (*A*), a superficial spreading melanoma (*B*), and a nodular melanoma (*C*), showing increased FLIP expression in malignant melanocytic skin lesions (*B* and *C*) as compared with benign ones (*A*). (*D*) Immunohistochemical staining of a nodular melanoma with an isotype-matched antibody. *Scale bar*: 100 μm.

nine atypical nevi. Taken together, only one of 32 (3%) benign melanocytic lesions expressed detectable, although weak, levels of FLIP.

FLIP expression in melanoma Immunohistochemical analysis of FLIP expression in melanomas revealed strong expression in six of eight (75%) superficial spreading melanomas (mean Breslow index 0.66 mm, range 0.25–1.25), nine of 10 (90%) nodular melanomas (mean Breslow index 5.17 mm, range 1.66–10.5), and nine of 11 (82%) cutaneous metastatic melanomas (**Figs 2**, **3**). Melanocytic expression of FLIP within the malignant melanocytic lesions was in all cases cytoplasmic as expected (**Fig 2**). In SSM and nodular melanomas, FLIP expression was less homogeneous throughout the tumor than in metastatic melanoma, where virtually all tumor cells were FLIP positive (**Figs 2, 4**). By morphometric analysis, FLIP staining was observed in 50% of SSM cells, 100% of nodular melanoma cells, and 100% of metastatic melanoma cells (**Fig 4**). It therefore clearly appears that the percentage of FLIP positive tumor cells within a melanocytic lesion increases with melanoma progression.

Melanoma cells overexpressing FLIP are more resistant to FasL and TRAIL To determine if the high levels of FLIP expression observed in malignant melanocytic lesions could account for resistance to death-receptor signaling and apoptosis, we transfected one of the rare human melanoma cell lines that we were able to identify as being sensitive to TRAIL and FasL-induced apoptosis, with a human FLIP expression vector. Four clones were selected by RT-PCR (**Fig 5*A***) and tested for sensitivity to recombinant TRAIL and FasL (**Fig 5*B***, ***C***). All FLIP-transfected clones were significantly more resistant to both TRAIL- and FasL-mediated apoptosis as compared with the mock-transfected WK melanoma cell line.

DISCUSSION

FLIP, a potent intracellular inhibitor of apoptotic cell death mediated by death receptor signaling, was originally discovered as a family of viral inhibitors of death receptor-mediated cell death found in several herpesviruses (including oncogenic HHV8/Kaposi's sarcoma associated herpesvirus and molluscipox virus). Cellular homologs of viral FLIP were subsequently identified, and shown to be expressed in certain tumor cell lines; however, involvement of FLIP in the resistance of tumor cell lines to Fas-mediated death is controversial because a correlation between FLIP expression and resistance to death receptor-mediated cell death was observed in some studies (Griffith *et al*, 1998), but not others (Ugurel *et al*, 1999; Zhang *et al*, 1999). Therefore the *in vivo* relevance of the data obtained from the study of tumor cell lines remains unclear.

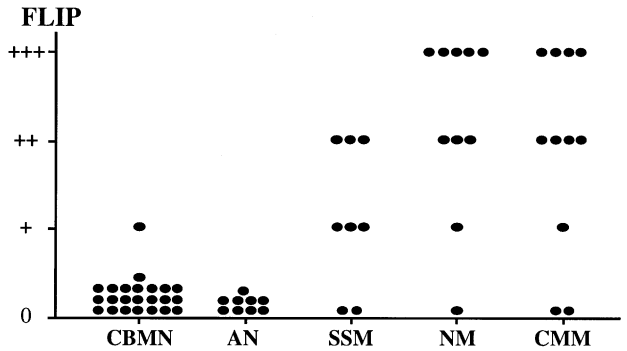

Figure 3. Semiquantitative analysis of FLIP expression in 61 benign and malignant human melanocytic skin lesions. Semiquantitative evaluation of the FLIP immunohistochemical staining intensity in the melanocytic component of common benign melanocytic nevi (CBMN), atypical nevi (AN), superficial spreading melanomas (SSM), nodular melanomas (NM), and cutaneous melanoma metastases (CMM). 0, no staining; +, weak staining; ++, moderate staining; +++, strong staining. Similar results were obtained using another rabbit antihuman FLIP antibody (AL148).

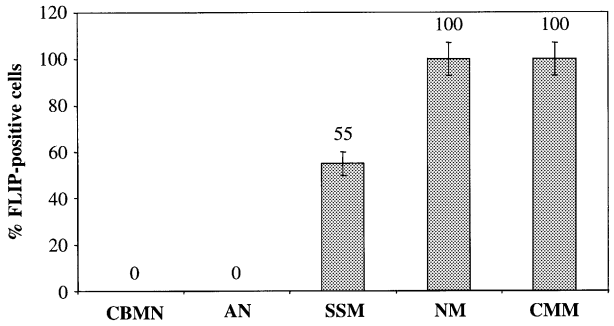

Figure 4. The percentage of cells expressing FLIP within melanocytic lesions increases with melanoma thickness. Percentage of FLIP-positive cells with respect to the total number of melanocytes within melanocytic lesions were analyzed by morphometry. CBMN, common benign melanocytic nevi; AN, atypical nevi; SSM, superficial spreading melanoma; NM, nodular melanoma; CMM, cutaneous melanoma metastases.

Here we provide data concerning the *in situ* expression of FLIP in human melanomas, and its potential relevance to tumor development. We show by immunohistochemical analysis of 61 benign and malignant human melanocytic skin lesions that FLIP is selectively overexpressed in malignant melanocytic lesions of the skin, namely superficial spreading melanomas, nodular melanomas, and cutaneous metastases of melanoma. FLIP expression was detected in only one of 32 benign melanocytic lesions, and at low levels. In contrast, expression was observed in 75% (six of eight) of superficial spreading melanomas, 90% (nine of 10) nodular melanomas, and 82% (nine of 11) of cutaneous melanoma metastases. Furthermore, within tumors, the percentage of cells expressing FLIP increased with progression, 50% of cells being FLIP-positive in SSM *versus* 100% in nodular melanoma and cutaneous metastatic melanoma. It clearly appears therefore that FLIP expression is more frequent in malignant melanocytic lesions, suggesting that it may, in certain cases, be implicated in the pathogenesis of melanoma, possibly by offering distinct advantages to the tumor cells. Here we show by transfection that overexpression of FLIP in melanoma cells renders them significantly more resistant to the pro-apoptotic effect of recombinant FasL and TRAIL.

Recent evidence has suggested that defective Fas-signaling due to loss of Fas expression or inhibition by FLIP, may be implicated in tumorigenesis. Indeed, loss of Fas expression or function has been demonstrated in several malignant tumors (Moller *et al*, 1994; Robertson *et al*, 1995; Lebel *et al*, 1996; Strand *et al*, 1996; Butler *et al*, 1998). Furthermore, loss of Fas expression has been shown to favor survival, proliferation, and eventually tumor formation in mouse models by preventing Fas-FasL mediated fratricide that occurs in three-dimensional settings if tumor cells express both Fas and FasL (Peli *et al*, 1999; Schroter *et al*, 2000). Likewise, in murine tumor models, inhibition of Fas signaling by overexpression of FLIP confers an advantage to tumors within an immunocompetent setting (Djerbi *et al*, 1999; Medema *et al*, 1999). Escaping death-receptor mediated cell death may therefore, at least in certain tumor types, enable tumors to grow in three dimensions and/or evade elimination by the immune system.

The resistance to FasL or TRAIL *in vitro* is a complex and multifaceted phenomenon, that at least *in vitro* does not simply correlate with a single factor, as for instance the basal level of FLIP. Nothwithstanding, our data showing strong FLIP expression *in vivo*, along with a correlation with loss of sensitivity to FasL and TRAIL after FLIP-transfection *in vitro*, may be of relevance *in vivo* given the mouse experiments referred to above and relatively new data showing that TRAIL mediates CD4 CTL target cell killing, including that of certain FasL resistant melanoma cells (Thomas and Hersey, 1998; Kayagaki *et al*, 1999). Inhibition of Fas and TRAIL-Receptor signaling by FLIP may therefore significantly inhibit the immune effector response to melanoma. Furthermore, taken together with the recent reports showing that the protein kinase C inhibitor bisindolylmaleimide sensitizes cells to Fas via down-regulation of FLIP expression (Willems *et al*, 2000; Zhou *et al*, 1999), pharmacologic modulation of FLIP expression may be of interest as a potential therapeutic approach for melanoma.

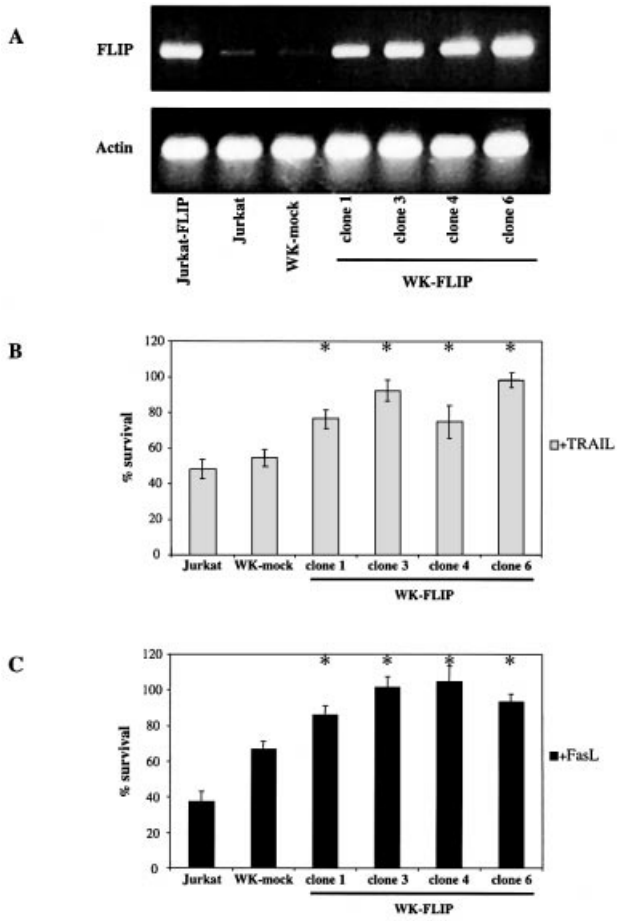

Figure 5. FLIP transfection enhances the resistance of melanoma cells to TRAIL and FasL. The TRAIL and FasL-sensitive melanoma cell line WK was transfected with an empty (WK-mock) or with a FLIP-containing expression vector (WK-FLIP). RT-PCR analysis of FLIP expression in FLIP-transfectants (*A*). Sensitivity (% survival) of established clones to TRAIL and FasL was tested after 16 h incubation with 100 ng per ml of recombinant human TRAIL (*B*) or FasL (*C*), respectively. Jurkat cells served as positive controls for TRAIL and FasL. Asterisks (*) denote FLIP-transfected clones for which the difference in sensitivity to TRAIL or FasL, as compared with the mock-transfected cell line, achieved statistical significance (t test, $p < 0.01$). Each clone was tested in quintuplicate, and experiments were performed three times. Results of one representative experiment are shown.

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