Inhibition of Growth of Melanoma Cells by CD95 (Fas/APO-1) Gene Transfer *In Vivo*

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Interaction of CD95 ligand with its cognate receptor CD95 induces apoptotic cell death. Alterations in this pathway within tumor cells can result in escape from apoptosis and from immune surveillance. Melanoma cells recently were found to escape an immune attack via high expression of CD95 ligand, thereby inducing apoptosis of activated T lymphocytes. When screening four human melanoma cell lines for expression of CD95 and CD95 ligand, respectively, an inverse correlation was found, i.e., cells expressing high levels for CD95 ligand (CD95L^{high}) were almost negative for CD95 and vice versa. Since coexpression of CD95 and CD95 ligand may lead to apoptosis by autocrine suicide or fratricide, it was tested whether overexpression of CD95 in CD95L^{high} melanoma cells results in apoptotic cell death. Upon transfection with a cytomegalovirus-promoter-driven expression vector encoding the CD95 gene, CD95L^{high} melanoma cells underwent apoptosis at a much higher level than CD95L^{low} melanoma cells. Apoptosis appeared to be due to the activation of CD95 as cell death was inhibited by cotransfection with a dominant negative mutant for the CD95 signaling protein, Fas-associated protein with death domain. Tumor progression of CD95L^{high} melanoma cells transplanted into nude mice was significantly reduced when recipient animals were injected with liposomes containing the CD95 expression vector. As demonstrated by immunohistochemistry and TUNEL staining, *in vivo* transfected tumor cells expressed CD95 and underwent apoptotic cell death. Hence, this study indicates that delivery of the CD95 gene inhibits tumor growth *in vivo* and thus might be a therapeutic strategy to treat tumor cells that express high levels of CD95 ligand. ***Key words: apoptosis/CD95/gene delivery/melanoma/therapy. J Invest Dermatol 115:1008–1014, 2000***

The incidence of malignant melanoma has been increasing in most countries over the last few decades. In contrast to nonmelanoma skin cancer, the pathogenesis of melanoma still remains an enigma on the whole. Intermittent and intense sun exposure, however, especially in childhood may be one of the essential factors. Malignant melanoma is one of the most aggressive tumors, causing for example approximately 6500 deaths per year in the U.S.A. (Johnson *et al*, 1995). Surgery is successful in primary melanoma, but disseminated disease is usually resistant to conventional treatment, which includes radiation and chemotherapy. Therefore there is an urgent need for new and innovative concepts to treat this malignant disease. Immunologic approaches including vaccination studies try to exploit the high antigenicity of melanomas and have begun to yield the first signs of success. Another promising approach in innovative cancer therapy is to activate the programmed cell death machinery in the tumor cells, thereby inducing apoptotic cell death (Korsmeyer and Greenberg, 1998).

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Abbreviations: CD95L, CD95 ligand; CMV, cytomegalovirus; FADD, Fas-associated protein with death domain; HVJ, hemagglutinating virus of Japan.

Apoptosis is not only essential for the development and the maintenance of tissue homeostasis but also serves as an effective mechanism by which harmful cells can be eliminated. Induction of apoptosis allows the organism to get rid of infected cells but also of tumor cells. Accordingly, resistance to apoptosis was identified as a crucial event in tumorigenesis. Apoptosis of tumor cells can be initiated by triggering cell death receptors, leading to activation of the intracellular apoptotic machinery (Korsmeyer and Greenberg, 1998). Among the many death receptors including TNF-R1, TRAIL-R1, TRAIL-R2, and DR-3, CD95, also called Fas or Apo-1, is one of the most potent transducers of apoptosis (Peter and Krammer, 1998). Triggering of the CD95 molecule either by agonistic antibodies or by the natural ligand, CD95L (FasL), induces apoptosis (Nagata, 1997). Ligand binding causes trimerization of CD95 and the trimerized cytoplasmic region then transduces the signal by recruiting the adapter molecule FADD (Fas-associated protein with death domain) which binds to CD95 via interaction of the death domain at its C terminus (Boldin *et al*, 1995; Chinnaiyan *et al*, 1995). The N-terminal region of FADD is responsible for downstream signal transduction by recruitment of the cysteine protease, caspase-8 (Muzio *et al*, 1996; Boldin *et al*, 1996). Subsequently activation of a cascade of downstream caspases executes apoptotic cell death (Alnemri *et al*, 1996). Hence, cells expressing high levels of CD95L can drive activated lymphocytes that bear CD95 on their surface into apoptosis and thereby escape an immune attack. Consequently, cells or organs expressing high

levels of CD95L are able to acquire a kind of immune privileged state (Bellgrau *et al*, 1995; Griffith *et al*, 1995).

Recently, it was shown that melanoma cells can escape an immune attack via expressing CD95L on their surface (Hahne *et al*, 1996). In metastatic lesions, CD95-expressing T lymphocytes were found in close proximity to the tumor cells. Upon incubation with CD95L-positive melanoma cells, apoptosis of CD95-sensitive targets occurred. In addition, CD95L-positive murine melanoma cells grew rapidly when transplanted into normal recipients, whereas tumor progression was delayed when they were transplanted into *lpr* mice which lack CD95. Hence, expression of CD95L may contribute to the immune privileged state of melanoma cells and thereby enable progressive growth. As demonstrated recently *in situ*, the expression of CD95L appears to be enhanced during progression of malignant melanoma (Maeda *et al*, 1998). High expression of CD95L on melanoma cells was also confirmed by other groups (Sprecher *et al*, 1999; Terheyden *et al*, 1999); however, in these cases no significant correlation between CD95L expression in melanoma cells and apoptosis in melanoma infiltrating mononuclear cells was found. High levels of CD95L expression were also found on cells derived from squamous cell carcinomas of the head and neck (Gastman *et al*, 1999), hepatocellular carcinomas (Strand *et al*, 1996), colon cancers (O'Connel *et al*, 1996), astrocytoma (Saas *et al*, 1997), and liver metastases of a colon adenocarcinoma (Shiraki *et al*, 1997).

Screening human melanoma cell lines for expression of CD95 and CD95L, respectively, we observed an inverse correlation of these two molecules, i.e., cells expressing high levels for CD95L ($CD95L^{high}$) were almost negative for CD95 and vice versa. Since it was previously shown that coexpression of CD95 and CD95L may lead to apoptosis by autocrine suicide or fratricide (Dhein *et al*, 1995; Friesen *et al*, 1996) we postulated that overexpression of CD95 in $CD95L^{high}$ melanoma cells should result in apoptotic cell death. Upon overexpression of CD95, $CD95L^{high}$ melanoma cells underwent apoptosis at a much higher level than $CD95L^{low}$ melanoma cells. Accordingly, tumor progression of $CD95L^{high}$ melanoma cells transplanted into nude mice was significantly reduced when recipient animals were injected with liposomes containing an expression vector encoding CD95. Hence, this study indicates that *in vivo* overexpression of CD95 might be a therapeutic strategy to treat tumor cells which express high levels of CD95L.

MATERIALS AND METHODS

Reagents Antibodies (polyclonal rabbit IgG) directed against CD95L and CD95, respectively, were purchased from Santa Cruz, Santa Cruz, CA. An expression vector for CD95 was constructed by subcloning cDNA encoding CD95 (kindly provided by S. Nagata, Osaka, Japan) into XhoI/XbaI sites of pBKCMV vector (Stratagene, La Jolla, CA), which contains a cytomegalovirus (CMV) promoter cassette. An expression vector of dominant negative inhibitor of FADD was a kind gift from V.M. Dixit, San Francisco (Chinnaiyan *et al*, 1995). An expression vector for cowpox serpin CrmA was obtained from K. Schulze-Osthoff, Münster, Germany. The broad spectrum caspase inhibitor z-Val-Ala-Asp-CH_2F (zVAD) was purchased from Enzyme Systems Products (Livermore, CA). pSVβ-galactosidase vector was purchased from Stratagene.

Cells The human melanoma-derived cell lines G361, MeWo, p22, and p38 were a kind gift from T. Horikawa, Kobe, Japan. Cells were maintained in Eagle's modified essential medium (MEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% antimycotic/antibiotic solution in a humidified atmosphere containing 5% CO_2.

Western blot analysis Cells were harvested by use of a rubber policeman and lyzed in RIPA buffer (10 mM Tris pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 4 μg per ml aprotinine, 1 mM sodium orthovanadate) for 15 min on ice. After centrifugation, supernatants were collected, and the protein contents were measured with a BioRad Protein assay kit (BioRad, Hercules, CA). Twenty micrograms of each protein sample were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, blotted to PVDF membranes (Immobilon-p, Bedford, MA), and incubated with antibodies directed against CD95 and CD95L, respectively. Signals were detected by use of an ECL kit (Amersham, Buckinghamshire, U.K.). To monitor equal loading of proteins, blots were reprobed with an antibody directed against α-tubulin (Calbiochem, Cambridge, MA).

Immunohistochemistry Cells were seeded on Chamber slides (Nunc, Roskilde, Denmark), fixed in 4% paraformaldehyde 24 h later, and washed three times in phosphate-buffered saline (PBS). Samples were then incubated with a rabbit polyclonal IgG directed against CD95L or against CD95 at 37°C for 80 min, washed three times in PBS at room temperature each for 5 min, and incubated with fluorescein-isothiocyanate (FITC) conjugated goat antibody directed against rabbit IgG (BioSource, Camarillo, CA) at 37°C for 30 min. Samples were then extensively washed five times in PBS at room temperature each for 5 min, and reactions were visualized under a fluorescence microscope.

Double immunostaining of melanoma cells implanted into nude mice was performed by using mouse monoclonal IgG to CD95 (Novus Molecular, San Diego, CA) and rabbit polyclonal IgG to CD95L (incubation for 1 h at 37°C). After extensive washing with PBS, rhodamine-conjugated antimouse IgG or FITC-coupled antirabbit IgG were added. Samples were evaluated under a fluorescence microscope using two different filters.

Transfection Cultured cells were cotransfected with 1 μg pSVβ-galactosidase vector along either with 1 μg of a CMV-promoter-driven expression vector of CD95 (pCMV-CD95) or with its insertless control vector (pBKCMV) using the Lipofectamine method (Life Technologies, Gaithersburg, MD). Forty-eight hours after transfection, cells were fixed in PBS containing 2% paraformaldehyde, 0.2% glutaraldehyde at 4°C for 20 min and stained for 5 h with X-gal (100 μg per ml), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM $MgCl_2$ in PBS. Positive cells were microscopically analyzed for apoptosis according to the method described by Bertin *et al* (1997) and Hsu *et al* (1995) with slight modifications (Aragane *et al*, 1998). Apoptotic cells were differentiated as blue rounded cells from intact living flat cells. Four random fields were counted. Data are given as the percentage of living cells calculated according to the formula (number of living cells per field divided by number of total cells per field) × 100.

***In vivo* gene delivery** To generate an *in vivo* melanoma model, 1×10^5 MeWo cells were suspended in 100 μl fresh Eagle's MEM, and injected subcutaneously into the back of nude mice ($n = 6$). When the diameter of tumors reached 5 mm, *in vivo* injection of plasmids incorporated into hemagglutinin virus of Japan (HVJ) liposomes was conducted. HVJ liposomes were prepared as described previously (Kaneda *et al*, 1988; Kaneda, 1994; Saeki *et al*, 1997). About 50 μg of plasmid DNA were entrapped in 10 ml of liposome suspension. Approximately 500 ng of either pBKCMV or pCMV-CD95 encapsulated into HVJ liposomes (100 μl) were directly injected into the tumors twice a week. Tumor size and weight of the mice were monitored once a week. Twenty-eight days after the first injection mice were sacrificed, and tumors were excised, weighed, and snap frozen in liquid nitrogen until being further processed.

TUNEL assay Tumor samples were fixed in 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 10 min. Samples were then rinsed in PBS three times. TUNEL staining was performed using In Situ Cell Death Detection kit™ (Boehringer Mannheim, Mannheim, Germany). Briefly, fixed samples were reacted with TUNEL reaction buffer containing TdT at 37°C for 60 min and washed three times at room temperature each for 5 min. Samples were evaluated under a fluorescence microscope.

RESULTS

Inverse correlation of the expression of CD95 and CD95L in different melanoma cell lines The constitutive expression of CD95L in different melanoma cell lines was determined by western blot analysis. Proteins were extracted from unstimulated MeWo, p38, G361, and p22 cells, and subjected to western blot analysis using an antibody directed against CD95L. As depicted in **Fig 1**, the melanoma cell lines MeWo and p38 expressed high levels of CD95L (*lanes 2* and *4*), whereas only minimal amounts were detectable in G361 and p22 cells (*lanes 1* and *3*). The inverse pattern was found for the expression of CD95: G361 and p22 cells expressed high amounts of CD95 protein, while MeWo and p38 did not. These data were confirmed by immunofluorescence

analysis which revealed strong staining for CD95L in MeWo and p38 cells, whereas G361 and p22 cells were almost negative (**Fig 2**). Hence, MeWo and p38 were referred to as $CD95L^{high}$ cells and G361 and p22 as $CD95L^{low}$ cells. Immunohistochemical staining using an antibody directed against CD95 revealed the same inverse expression pattern for CD95; melanoma cells expressing high levels of CD95L did not stain for CD95 and vice versa (**Fig 2**). The different expression of CD95 was also confirmed by treating the cells with recombinant CD95L. CD95L induced significant apoptosis in G361, but not in MeWo cells (data not shown). For the further studies, MeWo cells were used as $CD95L^{high}$ cells and G361 as $CD95L^{low}$ cells.

Induction of CD95 expression by transfection Since MeWo cells express high amounts of CD95L but only minimal numbers of CD95 receptors we were interested whether $CD95L^{high}$ cells undergo apoptosis upon expression of significant amounts of CD95. To address this question, a CMV-promoter-driven expression vector for CD95 was first transfected into MeWo and G361cells. To avoid possible apoptotic cell death by just transfecting the CD95 expression vector, in the initial studies an expression vector encoding the antiapoptotic cowpox serpin CrmA was cotransfected (Tewari *et al*, 1995). Cells were harvested 48 h later, proteins were extracted, and western blot analysis was performed using an antibody directed against CD95. As shown in **Fig 3**, significant levels of CD95 protein were detectable in both MeWo and G361 transfected with the CD95 encoding construct, while after mock transfection constitutive expression was only found in G361 (*lane 1*) but not in MeWo cells (*lane 3*).

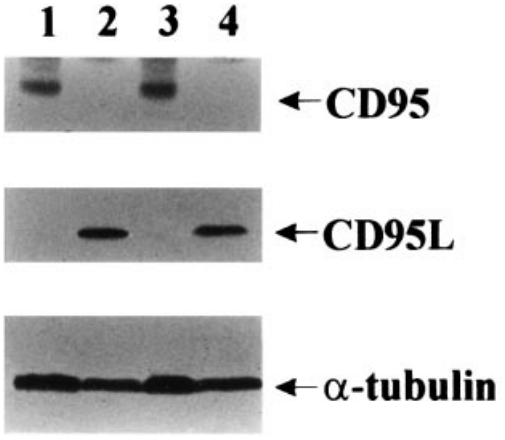

Figure 1. Inverse expression of CD95L and CD95 by human melanoma cells. Proteins were extracted from untreated G361 (*lane 1*), MeWo (*lane 2*), p22 (*lane 3*), and p38 cells (*lane 4*) and fractionated in 12% SDS-PAGE. Western blot analysis was performed using antibodies directed against CD95L, CD95, and α-tubulin.

Overexpression of CD95 causes stronger apoptosis in $CD95L^{high}$ cells than in $CD95L^{low}$ cells To address the initial question whether ectopical expression of CD95 leads to apoptosis of melanoma cells, the CD95 expression vector was transfected into MeWo and G361 cells. To determine the efficacy of transfection, an expression vector encoding β-galactosidase was cotransfected. Sixteen hours later cells were fixed and the viability of β-galactosidase-expressing cells was evaluated as described previously (Hsu *et al*, 1995; Bertin *et al*, 1997; Aragane *et al*, 1998). Mock-transfected cells did not change their morphology, still appearing dendritic. In contrast, the majority of CD95-transfected MeWo cells lost their original dendritic morphology and became rounded, indicative of apoptosis. In contrast, the apoptosis rate in CD95-transfected G361 cells was much less. Quantitative evaluation of apoptosis revealed a significantly enhanced death rate in CD95-transfected MeWo cells compared to CD95-transfected G361 cells (**Fig 4**).

To study whether sensitivity of CD95-transfection-induced apoptosis is dependent on the amounts of CD95 expressed, both $CD95L^{high}$ and $CD59L^{low}$ cells were transfected with increasing amounts of CD95 expression vector ranging from 0.25 to 1 μg. For comparison, we also used the human trichilemmoma cell line K-TL-1 (Kanzaki *et al*, 1981), which expresses only minimal amounts of CD95L as detected by immunohistochemical staining (data not shown). Upon transfection with 1 μg expression vector for CD95, almost all MeWo cells underwent apoptosis (note that only successfully transfected cells were counted), while only around 50% apoptotic cells were observed in the G361 group (**Fig 4**). Transfection with the insertless mock construct did not have any impact on cell viability (data not shown). Although the efficacy of the induction of apoptosis declined with decreasing amounts of the CD95 expression vector transfected, more than 80% of MeWo cells became apoptotic upon transfection with 0.25 μg. In contrast, only 25% apoptotic cells were observed in G361 and K-TL-1 cells. Hence, the susceptibility to CD95-transfection-induced apoptosis was significantly enhanced in $CD95L^{high}$ cells in comparison with $CD95L^{low}$ cells. These data indicate that the efficacy of inducing apoptosis by overexpressing CD95 is critically dependent on the amounts of CD95L expressed by the respective cells.

Apoptosis induced by transfection with CD95 is inhibited by CrmA, a dominant negative mutant of FADD, or zVAD To rule out the unlikely possibility that just the transfection of such high molecular weight DNA like CD95 is toxic and thus interferes with cell viability, MeWo cells were cotransfected with either an expression vector encoding CrmA or a dominant negative mutant of FADD. As demonstrated in **Fig 5**, CD95-mediated apoptosis induced by overexpressing CD95 was

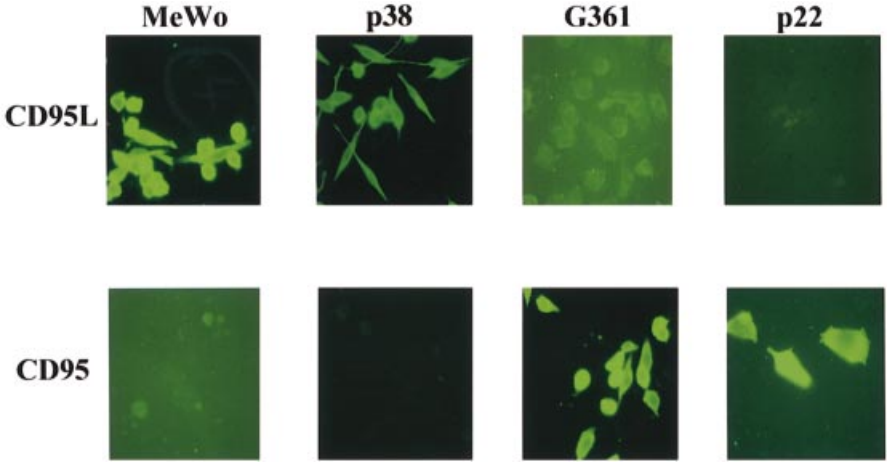

Figure 2. Inverse expression of CD95L and CD95 detected by immunohistochemistry. One day before immunostaining, cells (1×10^3) were seeded on chamber slides. After permeabilization of membranes, cells were stained by an indirect immunofluorescence technique using antibodies directed against CD95L and CD95, respectively. After incubation with FITC-labeled goat antirabbit IgG samples were evaluated by fluorescence microscopy.

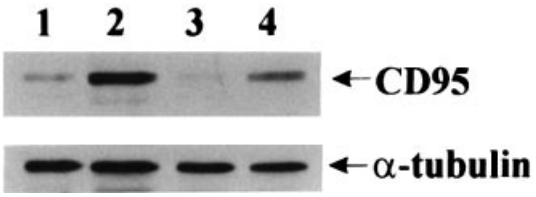

Figure 3. Expression of CD95 in MeWo and G361 cells. One day before transfection, cells were seeded on culture dishes at a density of 1×10^6. Twenty-four hours later, cells were transfected either with an expression vector encoding CD95 (*lanes 2*, *4*) or with an insertless control plasmid (*lanes 1*, *3*) using the Lipofectamine method. To avoid induction of apoptosis by overexpression of CD95, cells were cotransfected with an expression plasmid for CrmA. Forty-eight hours after transfection, proteins were extracted and subjected to western blot analysis using an antibody directed against CD95. *Lanes 1*, *2*, G361; *lanes 3*, *4*, MeWo.

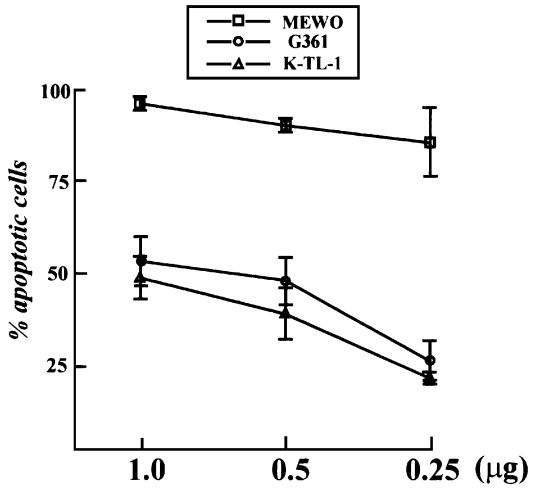

Figure 4. Cells expressing CD95L are more susceptible to apoptosis induced by overexpression of CD95. G361, MeWo, and KTL-1 cells were transfected with different concentrations of the CD95 expression vector. Cells were cotransfected with β-galactosidase and evaluated for apoptosis. Living dendritic-shaped blue cells and apoptotic rounded blue cells were counted in four independent microscopic fields and the percentage of apoptotic cells was calculated. Experiments were repeated three times; the data shown represent one of those.

significantly reduced by cotransfection with either CrmA or the dominant mutant of FADD. Accordingly, transfection-induced cell death was remarkably reduced by the broad spectrum inhibitor of caspases, zVAD.

In vivo* delivery of CD95 expression vector via HVJ liposomes reduces CD95L^{high}-expressing tumors** To elucidate whether transfection with CD95 inhibits growth of CD95L^{high} tumor cells *in vivo*, MeWo cells (1×10^5) were transplanted by subcutaneous injection into the back of nude mice. Approximately 1 wk later when tumors had reached 5 mm in diameter, the expression vector of CD95 (pCMV-CD95) or the insertless mock plasmid (pBKCMV) were injected directly into the tumors twice per week. To enable penetration into the cells, both plasmids were encapsulated into HVJ liposomes which have been reported to be effective in introducing foreign genes *in vivo* (Kaneda, 1999; Kaneda *et al*, 1999). The size of tumors was measured once a week. To monitor efficacy of *in vivo* transfection, a β-galactosidase expression vector was injected once into melanomas transplanted onto nude mice. Three days later, tumors were excised and β-galactosidase expression was visualized by staining tumor samples with X-gal. A significant number of melanoma cells expressed β-galactosidase as demonstrated by blue stained cells (**Fig 6*a, *insert*). While tumors grew progressively in the mock-injected group, introduction of the CD95 expression vector significantly delayed tumor growth as determined by tumor size.

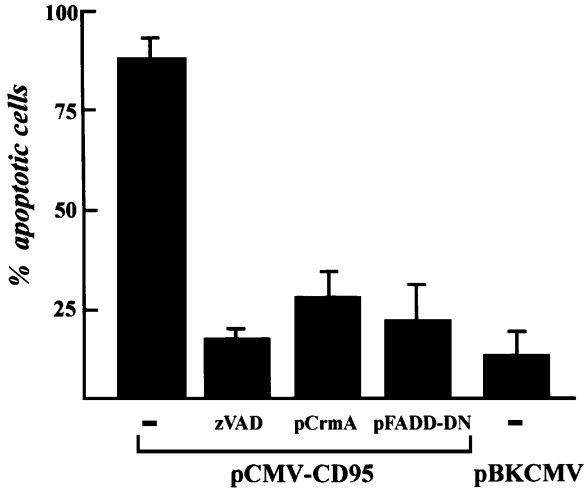

Figure 5. Inhibition of apoptosis induced by overexpression of CD95 in MeWo cells. MeWo cells (3×10^5) were transfected with an expression vector for CD95 along with constructs expressing either CrmA or a dominant negative mutant of FADD. In addition, cells transfected with CD95 only were cultured in the presence of 20 μM zVAD. Twenty-four hours later, cells were evaluated for apoptosis. Experiments were repeated three times; the data shown represent one of those.

Mice were sacrificed 28 d after the first injection, and tumors were excised and weighed. Tumors obtained from CD95-treated animals were significantly reduced in their weight compared with tumors of mock-treated animals (**Fig 6*b***).

To determine whether inhibition of tumor growth *in vivo* is due to induction of apoptosis via upregulated CD95, the CD95 expression vector was injected into MeWo tumors which had been transplanted into nude mice 7 d earlier. Tumors were excised 3 d after injection and double immunostaining using antibodies directed against CD95 and CD95L, respectively, was performed. In accordance with the *in vitro* data, *in vivo* transplanted MeWo cells expressed high levels of CD95L but not CD95 (**Fig 7**). In contrast, cells became CD95 positive upon injection of the CD95 plasmid, while CD95L expression was not affected. Together, these findings indicate that injection of CD95 plasmid encapsulated in HVJ liposomes is an effective method for expressing CD95 *in vivo*.

To address whether reduced growth is due to induction of apoptosis, samples were subjected to immunohistochemistry for CD95 and TUNEL staining. In mock-injected tumors in which only marginal expression of CD95 was found, no TUNEL-positive cells were detected (**Fig 8**). In contrast, in areas that were positive for CD95 due to injection of the CD95 plasmid the majority of cells became apoptotic as shown by TUNEL positivity. This indicates that *in vivo* injection of a CD95 expression vector into CD95L-expressing tumor cells inhibits tumor growth via induction of apoptosis.

DISCUSSION

Cancer cells escape immune clearance in several ways. These include avoidance of immune recognition by the loss of antigenic structures or by the downregulation of accessory molecules, or inhibition of an immune response by the release of immunosuppressive molecules including cytokines or gangliosides (Köck *et al*, 1991; Bergelson, 1993). Besides these evasive strategies tumor cells are able to actively disrupt an immune response. In this context, the expression of CD95L has been recognized as a novel mechanism to suppress an immune attack, since through this route tumor cells can deliver a death signal to CD95-positive target cells (Walker *et al*, 1997). Using melanoma cell lines, Hahne *et al* were the first to show *in vivo* that tumor cells use the expression of CD95L to escape immune rejection (Hahne *et al*, 1996).

Melanoma cells do not express high levels of CD95L in general, however. Of the four melanoma cell lines tested in this

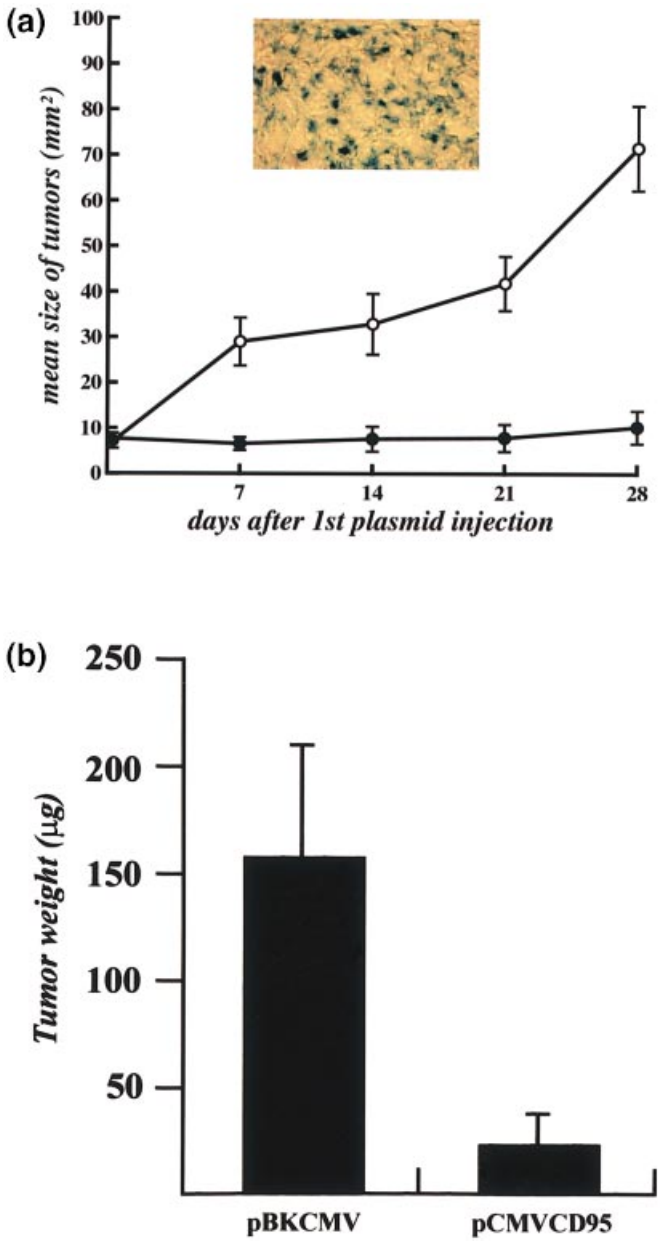

Figure 6. *In vivo* transfection with CD95 prevents tumor progression. MeWo cells (1×10^5) were transplanted into nude mice ($n = 6$) by subcutaneous injection. When tumors had reached the size of 5 mm in diameter, 0.5 μg pCMVCD95 (●) or 0.5 μg pBKCMV (○), both encapsulated into HVJ liposomes along with the β-galactosidase gene, were injected into the tumors twice a week. Efficacy on *in vivo* transfection was evaluated by staining tissue samples for X-gal (*a*, *insert*). Tumor sizes were measured at days 0, 7, 14, 21, and 28 (*a*). On day 28 tumors were excised and weighed. Tumor weights (mean ± SD) are shown on the *y* axis (mg) (*b*).

study only two (MeWo, p38) expressed CD95L, while the other two lines (G361, p22) were CD95L negative. Interestingly, the CD95L-positive melanoma cell lines did not express CD95. The absence of CD95 may represent an additional advantage for a tumor cell to survive and to progress (Schröter *et al*, 2000). The mutually exclusive expression of CD95 and CD95L observed in the four cell lines tested, however, must not be regarded as a general phenomenon since it could be representative only for selected cell lines. Ferrarini *et al* and Ugurel *et al* found coexpression of CD95 and CD95L on melanoma cells without any impact on cell survival. This resistance may be due to overexpression of antiapoptotic molecules (Ugurel *et al*, 1999) or alterations in the CD95-triggered signaling cascade (Ferrarini *et al*, 1999).

Several tumor cells including glioma, rhabdomyosarcoma, melanoma, colon cancer, and ovarian cancer (Yonehara *et al*, 1989; Morimoto *et al*, 1993; Weller *et al*, 1994; Owen-Schaub *et al*, 1998) were found to express CD95 at different levels. Such cells are principally susceptible for being killed through this pathway, e.g., by cytotoxic T cells that carry CD95L on their surface. In addition, they should be susceptible to therapies which target CD95, e.g., application of agonistic anti-CD95-antibodies (Weller *et al*, 1994). Hence, development of resistance to CD95-mediated apoptosis may be an important event during tumor progression. CD95 resistance can be achieved by secretion of soluble CD95 that traps CD95L (Cheng *et al*, 1994), by downregulation of CD95 (Weller *et al*, 1994), or by mutating CD95 (Shin *et al*, 1999). The latter possibility was recently reported by Shin *et al* who observed that somatic alterations of the CD95 gene in the cytoplasmic region might lead to the loss of its apoptotic function. In addition, loss or downregulation of CD95 expression may represent a further advantage for tumor cells which express high levels of CD95L, since coexpression of CD95 and CD95L may result in autocrine suicide or fratricide (Dhein *et al*, 1995). Certain chemotherapeutic drugs appear to make use of this pathway (Friesen *et al*, 1996). Taken together, enhanced expression of CD95L and loss of CD95 expression may support aggressive behavior of tumor cells.

The molecular mechanism by which MeWo and p38 cells downregulate CD95 expression remains to be determined. In this context it is important to mention that oncogenic Ras recently was found to downregulate CD95 at the transcriptional level, most likely through DNA methylation (Peli *et al*, 1999). This observation indicated that Ras not only promotes proliferation by its oncogenic activity, but also confers this effect by protecting cells from CD95L-mediated apoptosis.

When tumor cells express CD95 and provided that the signaling pathway is not altered, induction of apoptosis via the CD95 pathway appears as an attractive strategy to treat these cancers. Application of agonistic CD95 antibodies is problematic, however, as, due to the ubiquitous expression of CD95 on normal cells and tissues, injection of CD95 antibodies is toxic, e.g., due to fulminant hepatic failure (Lacronique *et al*, 1996). In addition, as mentioned above, many tumor cells lose the CD95 receptor on their surface during progression. Since coexpression of CD95 and CD95L can cause cell death either by autocrine suicide or fratricide (Dhein *et al*, 1995; Friesen *et al*, 1996), we were interested to study whether CD95 gene delivery is a feasible route to eliminate melanoma cells which express CD95L but not its cognate receptor CD95. To address this issue, we first introduced a CMV-driven expression vector encoding CD95 into MeWo and G361 cells. Spontaneous apoptosis was observed in both cell lines; however, the apoptosis rate was significantly higher in the transfected MeWo cells. Since G361 cells do not express CD95L, spontaneous apoptosis may be attributed just to the overexpression of CD95. Previous studies have demonstrated that mere overexpression of the CD95 receptor may suffice to cause apoptosis (Liu *et al*, 1996). As the apoptosis rate was significantly higher in MeWo cells, however, we anticipated that this is due to the interaction of ectopically expressed CD95 and constitutively expressed CD95L. To further support this anticipation, in preliminary experiments we tested whether transfected MeWo cells can be rescued by adding neutralizing anti-CD95 antibodies. Surprisingly, this approach did not alter the survival significantly. We do not have an explanation for this phenomenon yet; however, it may be that we have not yet found the optimal conditions concerning antibody concentrations or kinetics. Studies are ongoing to try to clarify this issue.

Nevertheless, the *in vitro* observations supported our hypothesis that CD95L expression may sensitize cells to undergo apoptosis following CD95 gene delivery. To test whether this approach is also effective *in vivo*, we transplanted MeWo cells into nude mice. Since nude mice are immunodeficient, human melanoma cells are not rejected and grow progressively. *In vivo* gene delivery has been

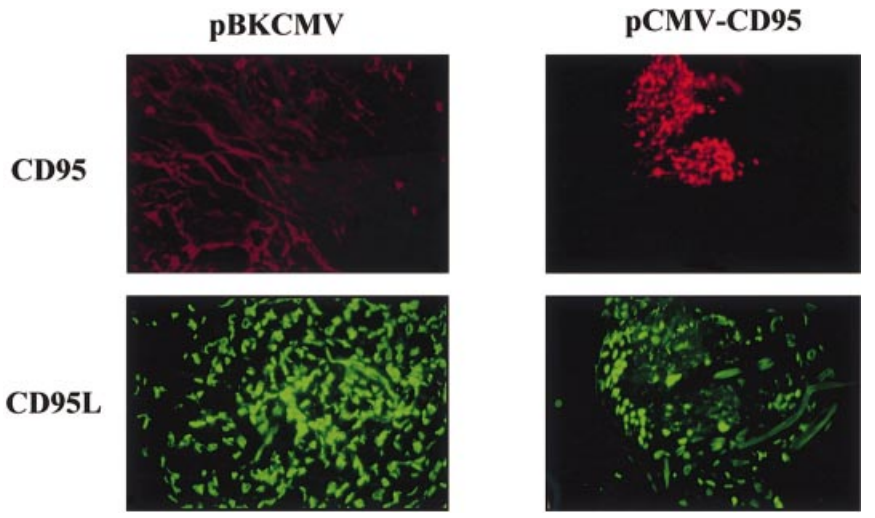

Figure 7. *In situ* detection of CD95 after *in vivo* transfection. MeWo cells (1×10^5) were transplanted into nude mice ($n = 6$) by subcutaneous injection. When tumors had reached the size of 5 mm in diameter, *in vivo* transfection with CD95 or with the mock construct was performed as described in **Fig 6**. Two days later, tumors were excised and immunohistochemistry was performed using an FITC-conjugated antibody against CD95L and a phycoerythrin-conjugated antibody against CD95.

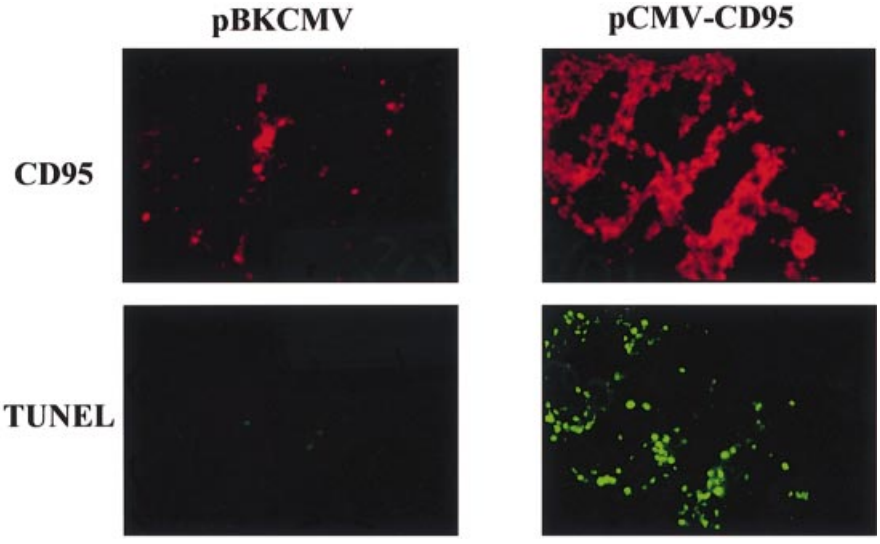

Figure 8. *In vivo* transfection of MeWo cells with CD95 results in apoptosis. MeWo cells (1×10^5) were transplanted into nude mice ($n = 6$) by subcutaneous injection. When tumors had reached the size of 5 mm in diameter, *in vivo* transfection with CD95 or with the mock construct was performed as described in **Fig 7**. Two days later, tumors were excised. Tissue sections were stained for CD95 and for apoptotic cells using the TUNEL method.

performed previously using several different methods (Bonnekoh *et al*, 1995; Hengge *et al*, 1995). We used the HVJ liposome method, which involves the entrapment of DNA and high-mobility group 1 protein within liposomes and the use of ultraviolet-inactivated HVJ to enhance liposome fusion with cell membranes. This procedure has already been utilized successfully for *in vivo* gene delivery to various types of tissue (Kaneda *et al*, 1988). Sawamura *et al* recently used the HVJ method to transfer the herpes simplex virus thymidine kinase gene into murine squamous cell carcinomas (Sawamura *et al*, 1997). Local injection of HVJ liposomes followed by administration of ganciclovir to mice resulted in tumor growth inhibition. As demonstrated in this study, $CD95L^{high}$ melanoma cells that were injected with liposomes containing the empty vector grew progressively, whereas tumor growth was completely stopped upon injection of liposomes containing the CD95 construct. *In vivo* delivery of the CD95 gene was successful since tumor cells located in the injected areas were found to be CD95 positive by immunohistochemistry. Inhibition of tumor growth appears to be due to the induction of apoptosis since numerous TUNEL-positive cells were detectable in the injected areas. Most of the TUNEL-positive cells also expressed CD95, indicating that apoptosis induced by transfer of the CD95 gene may be due to autocrine suicide.

Taken together, these data indicate, as already proposed recently (Schröter *et al*, 2000), that CD95 gene transfer may serve as a strategy to induce apoptosis selectively in tumor cells that express high levels of CD95L. Of course, a suicide gene therapy alone as used in this study would be insufficient to treat melanoma, since disseminated metastasis, the most critical aspect in terms of survival, would not be affected. Combination with other strategies, e.g., immunotherapeutic approaches, might overcome this problem. An alternative would be the systemic delivery of suicide genes like CD95 under control of promoters of melanoma-specific genes. Although these problems still remain to be solved, this study provides the proof in principle that *in vivo* delivery of the CD95 gene can be used to eradicate CD95L-expressing tumors. Accordingly, local injection of HVJ liposomes containing CD95 DNA could be useful for the treatment of disseminated cutaneous melanoma metastasis that cannot be removed surgically. One has to bear in mind, however, that this strategy will only work if the apoptotic pathway is not blocked, e.g., by overexpression of antiapoptotic molecules, like FLIP, bcl-2, bcl-x, or others.

It was recently shown that basal cell carcinomas, another kind of skin cancer, express high amounts of CD95L (Buechner *et al*, 1997; Gutierrez-Steil *et al*, 1998). Regression of basal cell carcinomas by intralesional interferon-α treatment seems to be due to upregulation of CD95, thereby causing CD95/CD95L-induced suicide (Buechner *et al*, 1997). Hence, CD95 gene delivery should have the same therapeutic effect. Liposomes are known to penetrate the corneal layer of the skin and to accumulate in the epidermis. As demonstrated recently for DNA repair enzymes (T4N5 endonuclease, photolyase), liposomes can deliver encapsulated proteins into

cells of the skin *in vitro* and *in vivo* (Yarosh *et al*, 1994; Kulms *et al*, 1999). Although it remains to be determined whether topically applied liposomes can be used to deliver genes *in vivo* into the skin as effectively as proteins, it is tempting to speculate on the therapeutic potential of topical CD95 gene delivery for the treatment of skin cancer by this route.

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