Selective Induction of Apoptosis in Melanoma Cells by Tyrosinase Promoter-Controlled CD95 Ligand Overexpression

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Induction of apoptosis has been demonstrated previously by overexpression of CD95 ligand (CD95L) in cultured human melanoma cells. For *in vivo* approaches based on CD95L, however, targeted expression is a prerequisite and tyrosinase promoters have been considered for selection. Luciferase reporter gene assays performed for a representative panel of melanoma cell lines characterized by strong (SK-Mel-19), moderate (SK-Mel-13, MeWo), weak (A-375), and missing expression (M-5) of endogenous tyrosinase revealed high tyrosinase promoter activities in SK-Mel-19, SK-Mel-13, and MeWo, but only weak activities in A-375 and M-5 as well as in non-melanoma cell lines. After transfection of a CMV promoter CD95L expression construct, melanoma cells were found highly sensitive, as compared with non-melanoma cells. By applying a tyrosinase promoter CD95L construct, apoptosis was selectively induced in SK-Mel-19, SK-Mel-13, MeWo as well as in A-375, which was characterized by high CD95 surface expression and high sensitivity to agonistic CD95 activation. M5 and non-melanoma cell lines remained uninfluenced. Also, resistance to agonistic CD95 activation seen in MeWo characterized by weak CD95 surface expression was overcome by overexpression of CD95L. Our investigations provide evidence that tyrosinase promoter CD95L constructs may be of value for selective induction of apoptosis in therapeutic strategies for melanoma.

Key words: apoptosis/CD95L/FasL/cell targeting/melanoma/tyrosinase promoter
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Melanoma is a highly malignant tumor developing from transformed melanocytes or nevus cells, and is characterized by increasing incidence within the last decades (Garbe and Blum, 2001). Its clinical malignancy results from its ability to form early metastasis as well as from its high resistance to chemotherapeutic drugs (Serrone and Hersey, 1999). The cytotoxic activity of chemotherapeutic compounds often coincides with the induction of apoptosis in malignant cells. It seems that chemotherapy resistance is related to defects in apoptotic signalling cascades and/or increased expression of anti-apoptotic proteins (Radetzki *et al*, 2002; Pommier *et al*, 2004) whereby apoptosis resistance of tumor cells may be overcome by strong expression of proapoptotic factors (Schulze-Bergkamen and Krammer, 2004).

CD95 ligand (CD95L/FasL) has been characterized as a death ligand produced by immune cells for triggering apoptosis in respective target cells (Krammer, 2000). It specifically binds on the cell surface and initiates formation of the death inducing signalling complex that activates both caspase cascades and mitochondrial cytochrome *c* release in melanoma cells (Eberle *et al*, 2003).

In a previous study, we have shown that induced expression of CD95L in human melanoma cell lines activates the CD95 signalling pathway and initiates apoptotic cell death *in vitro* and in xenotransplants (Eberle *et al*, 2003). Severe side-effects, however, such as liver toxicity have to be taken into consideration by using this system *in vivo*, as shown in mice treated systemically with agonistic CD95 antibodies (Ogasawara *et al*, 1993; Daniel *et al*, 2001). Therefore, selective expression of CD95L seems to be a necessary prerequisite when thinking of a CD95L-based gene therapeutic approach for melanoma.

Specific cell targeting of transgene expression is presently attempted either by vector targeting (Nettelbeck *et al*, 2004) or by means of cell-specific promoters (Xie *et al*, 2001; Zhu *et al*, 2004). Pigment gene promoters as for the pigmentation key enzyme tyrosinase (Sturm *et al*, 2001) seem to be well suited for melanoma due to high and restricted expression of pigment genes, and human or murine-derived tyrosinase promoters have been used for expression of suicide genes (Siders *et al*, 1998; Park *et al*, 1999; McCart *et al*, 2002; Rothfels *et al*, 2003) as well as for targeting oncolytic adenoviruses to melanoma (McCart *et al*, 2002; Nettelbeck *et al*, 2002).

The tyrosinase promoter constructs used generally consist of a basal tyrosinase promoter element fused to one or several copies of the distal tyrosinase enhancer that confers high promoter activity due to internal M-box elements. These contain binding sites for microphthalmia-associated transcription factor (MITF), a basic-helix-loop-helix-type leucine-zipper protein that contributes to specific activation of

Abbreviations: CD95L, CD95 ligand; MITF, microphthalmia-associated transcription factor

the tyrosinase promoter in pigment cells (Yasumoto *et al*, 1997).

In this study, we report specific induction of apoptosis in human melanoma cells based on tyrosinase promoter-controlled expression of CD95L whereby melanoma cells were found highly sensitive to overexpression of CD95L, as compared with other tumor cell lines. Apoptosis was induced also in melanoma cells with only weak tyrosinase promoter activity and resistance to agonistic CD95 activation was overcome by CD95L expression. Thus, tissue-specific induction of apoptosis by CD95L overexpression in combination with viral vectors may be considered for therapeutic strategies in melanoma.

Results

Exogenous tyrosinase promoter activity is restricted to pigmented melanoma cells Selective activity of the tyrosinase promoter was investigated in three melanoma cell lines with strong or moderate expression of endogenous tyrosinase mRNA (SK-Mel-19, SK-Mel-13, and MeWo), in a melanoma cell line with weak (A-375) and another without any expression of endogenous tyrosinase mRNA (M-5). Tyrosinase mRNA levels of melanoma cell lines had been determined previously (Eberle *et al*, 1995) and several non-pigmented, non-melanoma cell lines (MCF-7, PFSK-1, HeLa, and Hep-G2) were included in the study for comparison.

Luciferase reporter gene assays performed after transient transfection of luciferase constructs driven by the tyrosinase promoter, the CMV promoter or the SV40 promoter, respectively, showed significantly stronger activity of the CMV promoter as compared with the SV40 promoter in all cell lines with the exception of SK-Mel-13. The tyrosinase promoter was as strong or even stronger than the CMV promoter in two pigmented melanoma cell lines (SK-Mel-13 and SK-Mel-19) and it was at 6% of the CMV promoter in another (MeWo). In contrast, the tyrosinase promoter activity was only weak (less than 2% of the CMV promoter) both in non-melanoma cell lines and in the melanoma cell lines A-375 and M-5 characterized by weak or missing expression of endogenous tyrosinase mRNA (Fig 1). Thus, the tyrosinase promoter conferred selective expression of transgenes preferentially in melanoma cells with strong or moderate expression of endogenous tyrosinase mRNA.

Exogenous tyrosinase promoter activity in melanoma cells corresponds to endogenous expression of tyrosinase protein and of the transcription factor MITF For further characterization of the cell lines used the expression of endogenous tyrosinase as well as of the transcription factor MITF reported to regulate tyrosinase promoter activity were studied by Western blot analysis. Significant tyrosinase protein was detectable exclusively in SK-Mel-13, SK-Mel-19, and in MeWo with the highest expression found in SK-Mel-19 (Fig 2). These three cell lines were also positive for MITF expression. The two protein bands (60 and 55 kDa) detected for MITF had been reported to correspond to phosphorylation at serine 73 (Hemesath *et al*, 1998) Non-

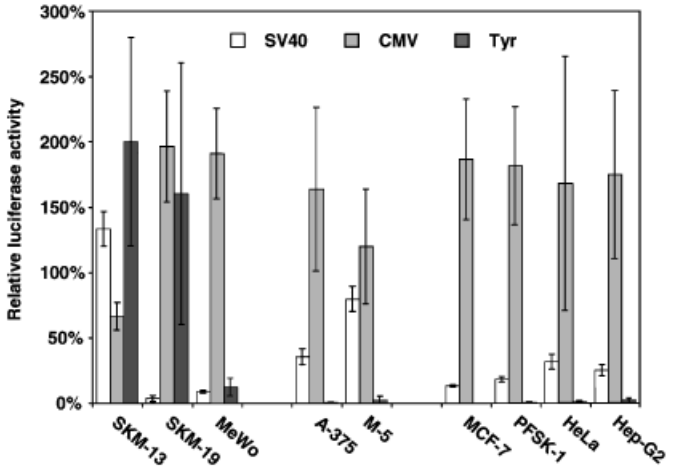

Figure 1
High tyrosinase promoter activity in melanoma cell lines characterized by high expression of endogenous tyrosinase mRNA. Relative luciferase activities (arbitrary units) were determined 48 h after transient transfection of melanoma cell lines SK-Mel-13, SK-Mel-19, MeWo, A-375, and M-5 as well as of non-melanoma cell lines MCF-7, PFSK-1, HeLa, and Hep-G2. Cells were transfected with luciferase reporter gene constructs under control of the SV40 promoter (*white bars*), the CMV promoter (*gray*) and the tyrosinase promoter (*black*). Relative luciferase activities were normalized by the median between the respective SV40 and CMV values. The median was set individually for each cell line to 100%. The complete experiment was performed three times for the tyrosinase promoter and for the SV40 promoter and was done twice for the CMV promoter, with each time triple values. Means and standard deviations calculated from the data of all experiments are presented here.

melanoma cell lines as well as melanoma cell lines A-375 and M-5 were negative for MITF expression indicating a dominant role of MITF for the activities of both the genomic

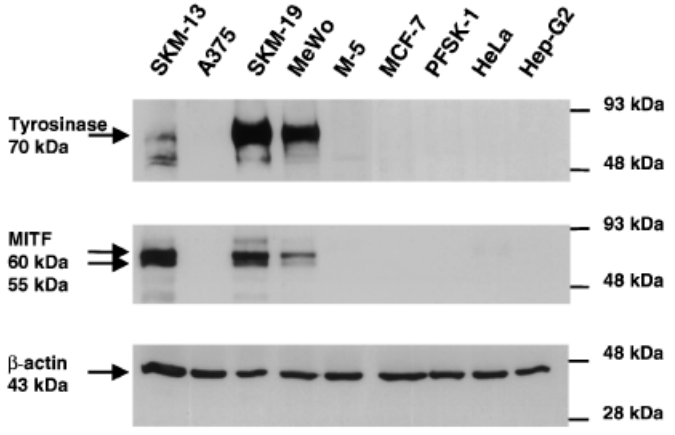

Figure 2
Correlated expression of tyrosinase and microphthalmia-associated transcription factor (MITF) proteins. Expression of tyrosinase and of MITF was determined by Western analysis for melanoma cell lines SK-Mel-13, SK-Mel-19, MeWo, A-375, and M-5 as well as for non-melanoma cell lines MCF-7, PFSK-1, HeLa, and Hep-G2. Expression of the 70 kDa tyrosinase protein was restricted to the melanoma cell lines SK-Mel-13, SK-Mel-19, and MeWo. In these three melanoma cell lines, also MITF expression was detectable as two prominent protein bands of 55 and 60 kDa, which were also seen in a normal human melanocyte control culture (data not shown). Equal protein amounts (40 μg per lane) were loaded and consistent blotting was confirmed by ponceau staining and by control analysis of β-actin. Western blot experiments were performed twice using independent cell isolates giving highly similar results.

tyrosinase promoter and the transfected tyrosinase promoter.

Melanoma and non-melanoma cell lines are sensitive for agonistic CD95 activation To investigate any responsiveness for CD95-mediated apoptosis of the cell lines used, we examined their CD95 surface expression and their sensitivity to CD95 activation by the agonistic antibody CH-11. By using FACS analysis relatively high CD95 surface expression was seen in most cell lines examined, whereas weak expression was found only in MCF-7 mammary carcinoma cells and in MeWo (Fig 3).

Also, apoptosis was inducible in non-melanoma and in most melanoma cell lines by CH-11 (Fig 4), whereby the melanoma cell lines A-375 and SK-Mel-19 were found highly sensitive and MeWo cells were largely resistant to CH-11-induced apoptosis.

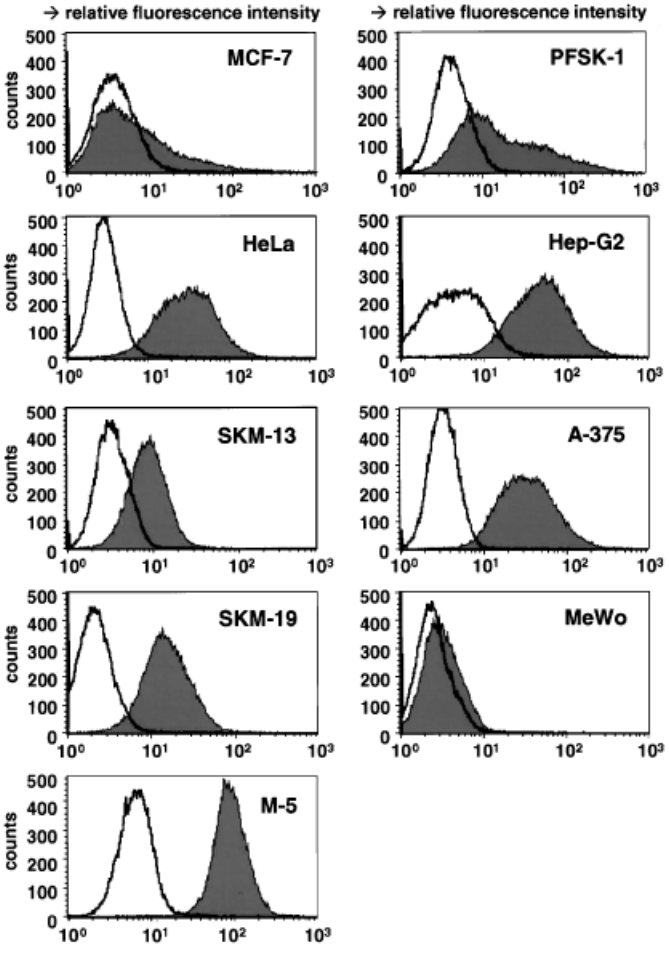

Figure 3
Melanoma and non-melanoma cell lines represent CD95 cell surface expression. CD95 surface expression was determined by FACS analysis in non-melanoma and melanoma cell lines. Cells were labelled with a specific CD95 antibody (*filled graphs*) and with an isotypic control antibody (*open graphs*), respectively. A shift indicating relatively high CD95 surface expression is seen in non-melanoma cell lines PFSK-1, HeLa and Hep-G2 as well as in the melanoma cell lines SK-Mel-13, A-375, SK-Mel-19, and M-5. Relatively weak CD95 surface expression was seen in MeWo melanoma cells and in MCF-7 mammary carcinoma cells. Western blot experiments were performed twice using independent cell isolates giving highly similar results.

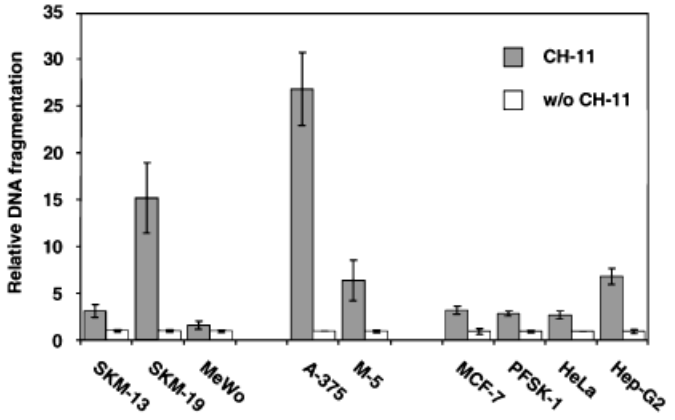

Figure 4
Melanoma and non-melanoma cell lines are sensitive to CD95-dependent apoptosis. Relative DNA fragmentation rates of melanoma and non-melanoma cell lines treated for 16 h with a CD95 agonistic antibody (CH-11) are shown (*gray bars*). For each cell line basic apoptotic rates of untreated controls were individually set to 1 (*white bars*). Clear induction of CD95-mediated apoptosis was seen in all cell lines ($p < 0.01$, Student's *t* test) with the exception of MeWo. The experiment was performed twice with both times triple values. Means and standard deviations of the values of both experiments are shown here.

Selective induction of apoptosis in melanoma cells by tyrosinase promoter-driven CD95L constructs To address the question whether apoptosis can be selectively induced in melanoma cells by cell type-specific expression of CD95L, we constructed vectors containing CD95L cDNA under control of a tyrosinase promoter (pTyr2-CD95L) and the CMV promoter (pIRES-CD95L). Transfection with pIRES-CD95L resulted in significant elevation of apoptosis both in melanoma and in non-melanoma cell lines (Fig 5*a*).

In contrast, transfection of pTyr2-CD95L resulted in efficient induction of apoptosis exclusively in melanoma cells (Fig 5*b*). Even higher apoptosis values as compared with the CMV promoter construct were seen in melanoma cell lines SK-Mel-13, SK-Mel-19, and MeWo. Interestingly, apoptosis induction was also high in A-375 indicating that weak tyrosinase promoter activity can be overcome by the high sensitivity of A-375 cells to CD95-dependent apoptosis. On the other hand, no significant induction of apoptosis was seen after transfection of pTyr2-CD95L in non-melanoma cells and in M5 (Fig 5*b*). Cytotoxicity levels determined in parallel as release of lactate dehydrogenase were increased in the cell lines showing apoptotic responses (data not shown).

To ascertain the relative number of dead cells in % after CD95L transfection, trypan blue assays as well as bisbenzimide nuclear stainings were performed for SK-Mel-13 and A-375 after transfection with the tyrosinase promoter CD95L construct and were compared with transfection with the control plasmid. The relative numbers were determined with 25% and 31% for SK-Mel-13 and with 7.4% and 10% for A-375, whereas transfection with the control plasmid resulted in less than 8% dead cells for SK-Mel-13 and less than 3% for A-375, respectively (Fig 6). Thus, highly selective and strong induction of apoptosis was shown to be achieved by tyrosinase promoter CD95L constructs in melanoma cells.

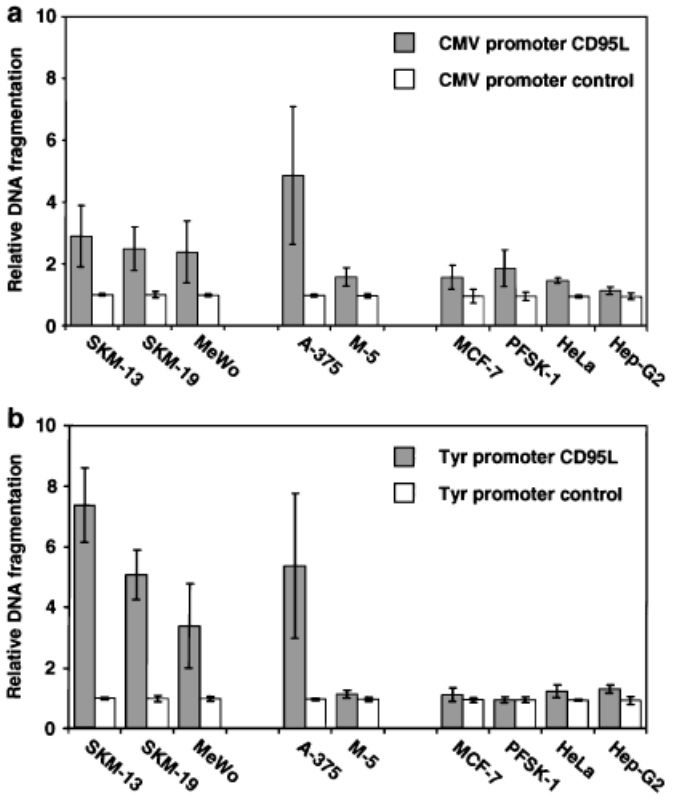

Figure 5
Selective induction of apoptosis in melanoma cells. Selective induction of apoptosis as determined by relative DNA fragmentation rates is shown in response to transient transfection of CD95 ligand (CD95L) expression constructs. (*a*) Relative DNA fragmentation rates of cell lines transiently transfected with a CMV promoter-CD95L construct (pIRES-CD95L; *filled bars*) as compared with a control plasmid (pIRES; *open bars*). Induction of apoptosis with the CMV promoter CD95L construct was statistically significant in all cell lines as compared with the control ($p < 0.02$, Student's *t* test). (*b*) Relative DNA fragmentation rates of cell lines transiently transfected with a tyrosinase promoter CD95L construct (pTyr2-CD95L; *filled bars*) as compared with a control plasmid (pTyrex-2; *open bars*). DNA fragmentation rates of cell lines transfected with the respective control plasmid were set individually to 1. The experiments were performed three times for each construct with each time triple values. Means and standard deviations of the values of all three experiments are given here. Induction of apoptosis in SK-Mel-13, SK-Mel-19, MeWo, and in A-375 with the tyrosinase promoter CD95L construct was statistically significant ($p < 0.01$, Student's *t* test).

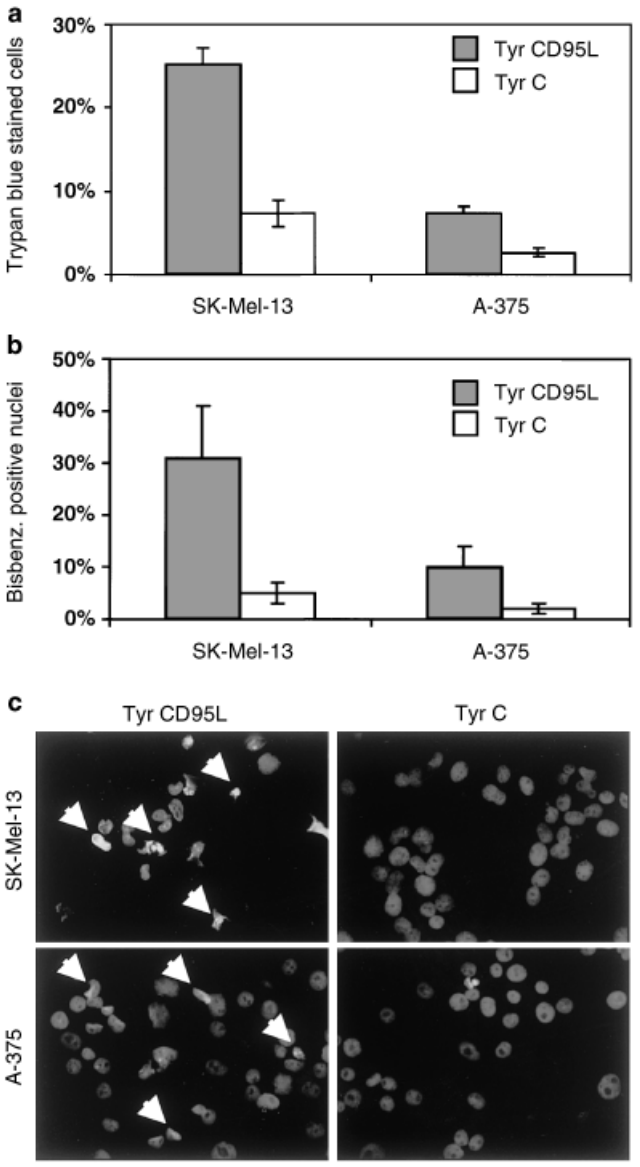

Figure 6
Determination of relative numbers of dead cells correlate with DNA fragmentation assays. Relative numbers of dead cells in melanoma cell lines SK-Mel-13 and in A-375 after transfection with the tyrosinase promoter CD95 ligand construct (Tyr CD95L) or with the control plasmid (Tyr C) were determined by typan blue staining (*a*) and by bisbenzimide nuclear staining (*b*). The experiments were performed in triplicate. Significant increase of dead and apoptotic cells, respectively, was found after transfection with the tyrosinase promoter CD95L construct as compared with the control ($p < 0.02$, Student's *t* test). (*c*) Cell pictures after bisbenzimide staining are shown, several positive cells are indicated.

After transfection of pIRES-CD95L, apoptosis induction was significantly stronger in most melanoma cell lines as compared with non-melanoma cells. To rule out weaker response of non-melanoma cells due to weaker transfection efficiency or weaker expression of CD95L, the expression of CD95L was monitored in two non-melanoma cell lines (MCF-7 and PFSK-1) after transient transfection of pIRES-CD95L and in SK-Mel-13, and no differences were found by Western analysis (Fig 7*a*). Also, the levels of CD95L surface expression were similar, as determined by FACS analysis (Fig 7*b*).

Activation of apoptotic cascades after overexpression of CD95L To investigate the signalling cascade after transfection of CD95L constructs, caspase-8, caspase-3 and caspase-7 were monitored at the protein level in SK-Mel-13 and A-375 cells. Overexpression of CD95L resulted in the reduction of procaspases 3 and 8 (data not shown), as well as in the formation of the 43/41 kDa caspase-8 intermediate fragments, the 18 kDa fragment of activated initiator caspase-8 and the 19/17 kDa fragments of activated main effector caspase-3. In addition, the 20 kDa fragment of active effector caspase-7 was found in A-375 clearly indicating activation of the caspase cascade after overexpression of CD95L (Fig 8). Since Bcl-2 proteins may play a critical role in the regulation of apoptosis, the levels of anti-apoptotic Bcl-2 and proapoptotic Bax were also investigated. A-375 cells were characterized by a reduced Bcl-2 to

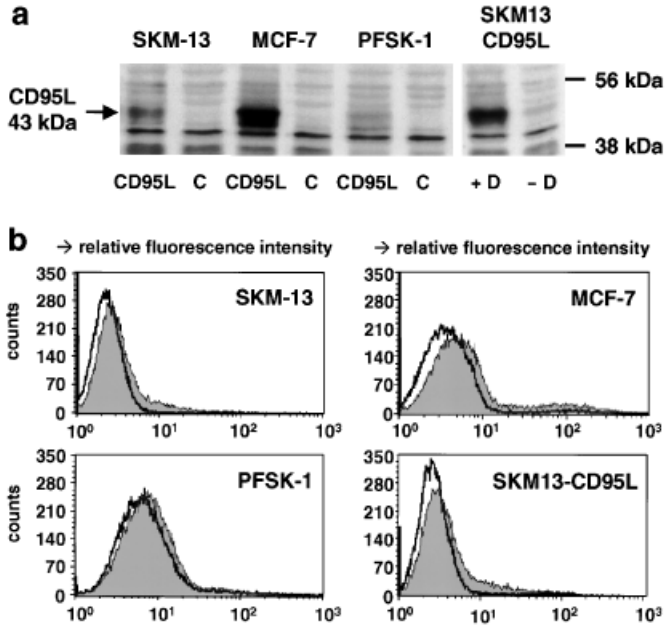

Figure 7
CD95L is expressed equally well in melanoma and non-melanoma cells after transient transfection. (*a*) Overexpression of CD95 ligand (CD95L) protein in SK-Mel-13, MCF-7 and PFSK-1 is shown by Western blot 48 h after transient transfection of pIRES-CD95L (CD95L). Vector-transfected cells are shown as negative controls (*C*). As positive control stably CD95L-transfected melanoma cells are shown (SKM13-CD95L) in which CD95L is inducible by doxycycline (+D), *versus* non-induced cells (−D). Equal amounts of proteins were loaded (40 μg per lane) and consistent blotting was confirmed by ponceau staining. For each cell line double experiments from independent cultures have been performed, giving highly similar results. (*b*) Surface expression of CD95L is shown 48 h after transient transfection of pIRES-CD95L (*filled graphs*) as determined by FACS analysis. Cells transiently transfected with a control plasmid (pIRES) were used as negative controls (*open graphs*). As a positive control, doxycycline-induced SKM13-CD95L cells (*filled graph*) are shown versus non-induced SKM13-CD95L cells (*open graph*). The overlay of the histograms of induced SKM13-CD95L cells *versus* the control revealed a shift to higher fluorescence in induced cells. This shift indicating CD95L surface expression is also seen in the overlays of transiently transfected melanoma and non-melanoma cells *versus* their respective controls.

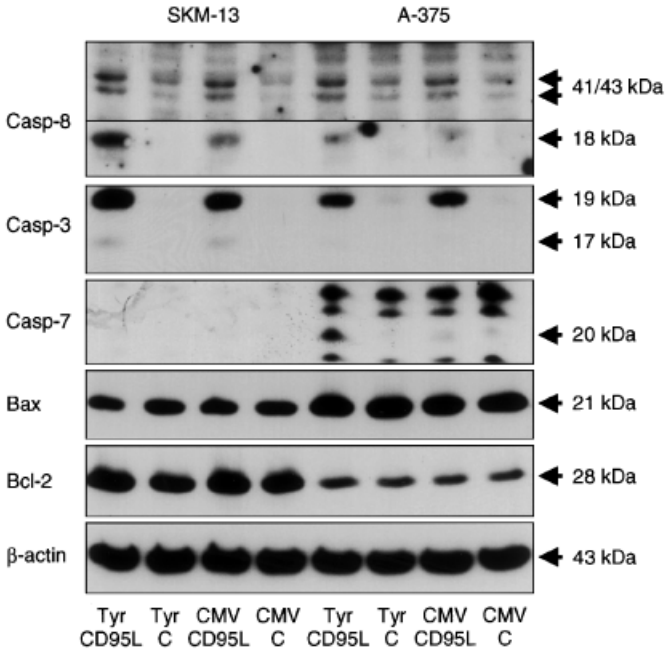

Figure 8
Caspase activation and protein levels of Bcl-2 and Bax. Western blots for capase-8, caspase-3, caspase-7, Bcl-2, and Bax are shown for melanoma cell lines SK-Mel-13 and A-375. Proteins were extracted 48 h after transient transfection of cells with either pTyr2-CD95L (Tyr CD95 ligand (CD95L)), with pIRES-CD95L (CMV CD95L) or the respective control plasmids without the CD95L cDNA (Tyr C; CMV C). Identical protein amounts (25 μg per lane) were loaded in each lane, consistent blotting was confirmed by ponceau staining and by detection of β-actin. Molecular weights of caspase cleavage products (Casp), Bcl-2, Bax and β-actin are indicated on the right side. For each cell line double experiments from independent cultures have been performed, giving highly similar results.

Bax ratio as compared with SK-Mel-13 possibly meaningful for the high sensitivity of these cells (Fig 8).

Discussion

Resistance to apoptotic stimuli contributes to disturbed homeostasis in cancer leading to predominance of cell proliferation *versus* cell death (Fulda and Debatin, 2003). Apoptotic resistance may be overcome by overexpression of proapoptotic genes, as shown previously for Bcl-X_S and CD95L in melanoma cells (Eberle *et al*, 2003; Hossini *et al*, 2003). For CD95-induced apoptosis, however, severe side effects such as liver toxicity have been described in nude mice treated systemically with agonistic anti-CD95 antibodies to inhibit the growth of xenotransplants (Ogasawara *et al*, 1993; Daniel *et al*, 2001). In consequence, cell type-specific gene expression seems to be a necessary prerequisite when thinking of anti-tumor approaches based on overexpression of death ligands.

Tyrosinase is the key enzyme of melanin synthesis and its expression is tightly restricted to melanocytic cells (Sturm *et al*, 2001). In accordance with the previous data on the activity of the tyrosinase promoter used (Park *et al*, 1999; McCart *et al*, 2002) we found high activity of a tyrosinase promoter reporter gene construct in melanoma cells after transient transfection, whereas, its activity was neglectable in non-melanoma cells. In addition, tight correlation of exogenous tyrosinase promoter activity with the endogenous expression of tyrosinase in melanoma cells was seen; melanoma cell lines characterized by high expression of endogenous tyrosinase mRNA and of tyrosinase protein showed high levels of activity, whereas other melanoma cells were characterized by only weak tyrosinase promoter activities.

With respect to the regulation of tyrosinase expression, several isoforms of the transcription factor MITF have been described as basic-helix-loop-helix-leucine-zipper proteins (Saito *et al*, 2002) of which the M-isoform is expressed in neural crest-derived melanocytes and binds to M-box elements in the tyrosinase promoter enhancer (Yasumoto *et al*, 1997). It plays an important role in melanocyte differentiation (Goding, 2000) and may also drive high Bcl-2 expression required for survival of normal melanocytes (McGill *et al*, 2002). Here, we found strong correlation of MITF and tyrosinase protein expression in melanoma cell lines. This may further indicate that exogenous tyrosinase promoter

activity depends on MITF-M activity in target cells (Yasumoto *et al*, 1997; Vachtenheim and Drdova, 2004) being of some importance for the applicability of these promoters in melanoma therapies. Tyrosinase promoters may be ineffective in amelanotic melanomas with no MITF expression.

Selective targeting of toxic genes encoding *Herpes simplex* virus thymidine kinase, *Escherichia coli* purine nucleoside phoshorylase or the diphteria toxin A chain to melanoma cells by applying tyrosinase promoters has been demonstrated in several studies (Siders *et al*, 1998; Park *et al*, 1999; McCart *et al*, 2002; Rothfels *et al*, 2003). As compared with toxic genes, the anti-tumor effect of proapoptotic genes is supposed to produce a more physiological response (Xie *et al*, 2001; Proskuryakov *et al*, 2003). In the present investigation we found that the combination of the CD95L with a tyrosinase promoter is able to selectively induce apoptosis in human melanoma cells by activation of the caspase cascade. On the other hand, when applying a constitutive CMV control promoter, apoptosis was induced both in melanoma and in non-melanoma cell lines. Transgenic CD95L expression was unequivocally verified at the protein level and was also proven as surface expression. Using the tyrosinase promoter, the apoptotic response in some pigmented melanoma cells was as high or even higher compared with the CMV promoter, which is often used for transgene expression due to its high activity (Xu *et al*, 2002).

Although non-melanoma cells were clearly less sensitive to CD95L plasmids, there was significant induction of apoptosis in MCF-7, PFSK-1, and in HeLa cells after CMV promoter-driven overexpression of CD95L. In general, however, melanoma cell lines revealed clearly higher sensitivity to ectopic CD95L overexpression as demonstrated by CMV promoter CD95L constructs, thus indicating the high potential and effectiveness of ectopic CD95L overexpression especially for melanoma cells. Furthermore, even weak tyrosinase promoter activities turned out to be sufficient for inducing apoptosis by CD95L expression provided that the cell line used was sensitive to CD95L. This has been demonstrated for A-375 characterized by weak expression of endogeneous tyrosinase mRNA shown previously (Eberle *et al*, 1995) but low Bcl-2/Bax ratio (Raisova *et al*, 2001), high CD95 surface expression, and highest susceptibility to CD95-triggered apoptosis as shown here.

Apoptosis resistance may counteract death receptor-based therapeutic approaches in tumor cells (Kamarajan *et al*, 2003). Also, downregulation of death receptors was shown to prevent signalling of death ligands in the TRAIL system (Yang *et al*, 2003). In our experiments resistance of melanoma cells was overcome by tyrosinase promoter-driven overexpression of CD95L in the cell line MeWo, which was found resistant to agonistic CD95 antibodies and which is characterized by high Bcl-2/Bax ratio (Raisova *et al*, 2001) and only weak CD95 surface expression, thus demonstrating the high potential of ectopic overexpression of death ligands.

In summary, our data provide evidence that combination of CD95L with tyrosinase promoters is an effective strategy for targeting of melanoma cells, due to selectivity of tyrosinase promoters and high sensitivity of melanoma cells to ectopic CD95L overexpression. As shown previously, CD95L also can induce a bystander effect via its soluble form and can sensitize melanoma cells for chemotherapeutics (Eberle *et al*, 2003). Anti-tumor gene therapeutic approaches are still hampered by limitations in transduction efficiencies in tumor cells (Heise and Kirn, 2000; Zhang *et al*, 2002). New vector systems such as replication-competent adenoviruses may represent the urgently needed breakthrough in this field (Zhang *et al*, 2002; Fechner *et al*, 2003; Peter *et al*, 2003; Nettelbeck *et al*, 2004). Once efficient vector systems are available, combined use of tyrosinase promoters and CD95L appears as a suitable strategy for apoptosis-based gene therapeutic approaches in melanoma.

Materials and Methods

Cell culture Five human melanoma cell lines were used in the present study: SK-Mel-13 (Carey *et al*, 1976), A-375 (Giard *et al*, 1973), SK-Mel-19 (Carey *et al*, 1976), MeWo (Bean *et al*, 1975), and M-5 (Liao *et al*, 1975). SK-Mel-19 had been characterized previously by high endogenous expression of tyrosinase mRNA. SK-Mel-13 and MeWo showed moderate tyrosinase mRNA expression, A-375 was characterized by only weak expression and M5 was completely negative for tyrosinase mRNA (Eberle *et al*, 1995). In addition, the breast adenocarcinoma cell line MCF-7 (Soule *et al*, 1973), the neuroectodermal tumor cell line PFSK-1 (Fults *et al*, 1992) the cervical carcinoma cell line HeLa (Scherer *et al*, 1953), and the hepatoma cell line Hep-G2 (Zannis *et al*, 1981) were examined. Cells were grown at 37°C and 5% CO_2. HeLa cells were maintained in RPMI medium (Biochrom, Berlin, Germany), Hep-G2 cells were maintained in MEM medium (Biochrom), and all other cell lines were maintained in DMEM (4.5 g per l glucose; Invitrogen, Karlsruhe, Germany). All growth media were supplemented with 10% fetal calf serum and antibiotics (Biochrom).

The agonistic anti-CD95 antibody CH-11 (IgM mouse; Beckman Coulter, Krefeld, Germany) was applied for 16 h in a concentration of 0.25 μg per mL. As a positive control for CD95L expression, stably transfected SKM13-CD95L cells were used, in which CD95L is inducible by doxycycline (Eberle *et al*, 2003). The experiments performed in this study have been approved by the Charité-Universitätsmedizin Berlin.

Plasmid constructs A CMV promoter CD95L construct (pIRES-CD95L) resulted from subcloning of the full-length mouse CD95L cDNA fragment (nucleotides 31–870, according to the sequence described by Lynch *et al*, 1994) into the *Not*I and *Eco*RI sites of pIRESneo (Promega, Mannheim, Germany). The tyrosinase promoter used consisted of the basal element of the human tyrosinase promoter and two upstream enhancer elements of the murine tyrosinase promoter (Park *et al*, 1999). A tyrosinase promoter CD95L construct (pTyr2-CD95L) resulted from subcloning of the full-length mouse CD95L fragment into *Bam*HI and *Eco*RI sites of pTyrex-PNP2 (Park *et al*, 1999) after the removal of the PNP gene. Luciferase reporter gene assays were performed with pTyrex-2 (Park *et al*, 1999) enclosing a tyrosinase promoter in front of the luciferase gene, pGL3c enclosing a SV40 promoter in front of the luciferase gene (Promega), and pAd5CMV-Luc enclosing a CMV promoter in front of the luciferase gene. pAd5CMV-luc has been constructed by insertion of a luciferase cDNA fragment into pZS2 (Fechner *et al*, 1999). Plasmid DNA for transfection experiments of human cell lines was routinely isolated using the Endofree Plasmid Maxi Kit (Quiagen, Hilden, Germany).

Transient transfection Human melanoma and non-melanoma cells were seeded in six-well plates with 2×10^5 cells per well. For transient transfection on the next day, cells at a confluence of 50% were washed with serum-free Opti-MEM medium (Invitrogen) followed by 4 h incubation at 37°C with 1 mL per well of the

respective transfection solution in Opti-MEM. Subsequently the transfection solution was removed, and cells were incubated for additional 44 h in their respective culture medium, before analysis. In order to obtain comparable transfection efficiencies, the lipid DMRIE-C (Invitrogen) and plasmid concentrations in the transfection solution had been adapted for each cell line according to luciferase values obtained after transient transfection with pGL3c. Volumes of DMRIE-C and amount of pGL3c plasmid used per 1 mL transfection solution: 1 μL/0.02 μg (SK-Mel-13), 1 μL/0.2 μg (MCF-7, PFSK-1), 1 μL/0.3 μg (A-375, M-5, HeLa), 2 μL/0.2 μg (Hep-G2), 2 μL/0.3 μg (MeWo), 4.2 μL/0.4 μg (SK-Mel-19). Other plasmids were used in equimolar amounts.

Luciferase assay After transient transfection in six-well plates, firefly luciferase activity was determined after 48 h using a luciferase assay system (Promega). Wells were rinsed with phosphate-buffered saline (PBS) and cells were scraped in 1 mL of reporter lysis buffer. Cellular debris was then removed by centrifugation (10,000 × *g*), 2 μL of each lysate were mixed with 50 μL of luciferase assay reagent buffer in eppendorf tubes, and light emission was determined in a Liquid Scintillation Analyzer Tricarb 1600TR (Canberra Packard, Frankfurt, Germany). Background emission (reagent buffer) was subtracted, and relative luciferase activities were calculated.

Quantification of apoptosis and cytotoxicity Apoptosis was quantified after transient transfection of CD95L constructs and control plasmids after 48 h by using a cell death detection ELISA (Roche Diagnostics, Mannheim, Germany), which detects mono- and oligonucleosomes formed in apoptotic cells. In parallel to apoptosis, relative cytotoxicity levels were determined by measuring lactate dehydrogenase activity in the culture fluids using a cytotoxicity detection system (Roche). For both assays the standard protocols of Roche have been applied. Relative apoptosis and cytotoxicity rates after transfection of CD95L constructs were calculated by normalization with the respective control plasmids (pIRES and pTyrex-2, respectively). Statistical significance was determined by Student's *t* test.

For trypan blue staining of dead cells and bisbenzimide staining of apoptotic nuclei, cells were harvested 48 h after transfection by trypsin-EDTA treatment and were pooled with spontaneously detached apoptotic cells collected before. After 1 × washing with PBS, a cell aliquot was mixed with an equal volume of trypan blue solution (0.4% in 0.8 M NaCl; Sigma-Aldrich, Taufkirchen, Germany). A minimum of 500 cells were counted for each transfection experiment performed in triplicate. Relative numbers of dead cells were calculated as the percentage of blue cells to total cell counts.

Other cell aliquots were applied for cytospins (30,000 cells each). For bisbenzimide staining, cytospins were fixed in 4% formaldehyde (methanol-free) for 30 min at 4°C and washed 2 × with PBS. Cells were permeabilized by incubation for 5 min in a 0.2% Triton X-100/PBS solution, washed 2 × with PBS and stained with Hoechst-33258 dye solution (1 μg per mL in PBS; Sigma) for 15 min at room temperature. After again washing 2 × with PBS, cells were mounted (MoBiTec, Göttingen, Germany) and examined by fluorescence microscopy. Apoptotic cells were identified by condensed or by fragmented nuclei. For quantification, a minimum of each 500 cells were counted for each transfection experiment performed in triplicate. The ratio of apoptotic cells was calculated as percentage of cells with condensed nuclei to total cell counts.

CD95L and CD95 surface expression analyses For analysis of CD95L surface expression, cells were transiently transfected with CD95L expression plasmids and control plasmids, respectively, and were harvested from culture plates by treatment with 0.02% EDTA in PBS. After washing with PBS, aliquots of 1×10^6 cells in 100 μL PBS, 1% BSA were incubated with phycoerythrin-labelled monoclonal CD95L antibody (clone MFL3; sc-19986PE, Santa Cruz, Heidelberg, Germany), as recommended by the manufacturer. After washing with PBS, surface expression was determined with a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). The mean fluorescence index was calculated using CellQuest software (Becton Dickinson).

CD95 surface expression was detected by FACS analysis of untransfected cells incubated either with a phycoerythrin-labelled monoclonal CD95 antibody (clone DX2; Becton Dickinson) as compared with an isotypic labelled IgG_1 monoclonal control antibody (clone MOPC-21, Becton Dickinson).

Western blot analysis For protein extraction, cells at a confluence of 60%–80% were washed with PBS and then immediately lysed in the respective lysis buffer. Lysis buffer for Bax, Bcl-2, MITF and tyrosinase: 10 mM Tris-HCl, pH 7.5, 144 mM NaCl, 1% Nonidet P-40, 0.5% sodium dodecyl sulfate (SDS), 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg per mL trasylol (Bayer, Leverkusen, Germany), 20 μM leupeptin, and 10 μM pepstatin. Lysis buffer for caspase analysis: 50 mM PIPES/HCl, pH6.5, 2 mM EDTA, 0.1% Chaps, 40 μM leupeptin, 15 μM pepstatin, 10 μg per mL trasylol, and 5 mM DTT (New England Biolabs, Frankfurt am Main, Germany). Lysis buffer for CD95L: 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 400 μM PMSF, 8 μM leupeptin, 6 μM pepstatin, 0.1% SDS, and 0.1% Triton X-100.

For Western blotting, cell lysates were homogenized and centrifuged for 5 min at 10,000 × *g*. Equal protein amounts were separated by electrophoresis under reducing conditions on 12% or 15% SDS-polyacrylamide gels in parallel with a broad range prestained SDS-PAGE protein standard (BioRad, München, Germany). Transfer of proteins to blotting membranes, ponceau staining and immunodetection of transferred proteins were performed as described previously (Eberle *et al*, 2003).

The following antibodies have been used: CD95L (rabbit polyclonal IgG, N-20, sc-834, Santa Cruz, 1:250); caspase-3 (mouse monoclonal IgG, 9661, New England Biolabs; 1:1000); caspase-8 (mouse monoclonal IgG, 9746, New England Biolabs; 1:1000); caspase-7 (mouse monoclonal IgG, 9492, New England Biolabs; 1:1000); Bcl-2 (mouse monoclonal IgG, sc-509, Santa Cruz, 1:200); Bax (rabbit polyclonal IgG, sc-493, Santa Cruz; 1:400); MITF (mouse monoclonal IgG Ab-3, clone C5 and D5, Biocarta, Hamburg, Germany, 1:200); tyrosinase (mouse monoclonal IgG, clone T311, Oncogene, San Diego, California, 1:1000); β-actin (mouse monoclonal, clone AC-15, Sigma-Aldrich, 1:10000). Two secondary horseradish peroxidase-labelled antibodies were used (HRP-labelled goat anti-rabbit immunoglobulins and HRP-labelled goat anti-mouse immunoglobulins, both from DakoCytomation, Hamburg, Germany, 1:5000).

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