Resistance to CD95/Fas-induced and ceramide-mediated apoptosis of human melanoma cells is caused by a defective mitochondrial cytochrome *c* release

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Abstract Intracellular CD95/Fas-signaling pathways have not been investigated in melanoma yet. Two different CD95 receptor-induced apoptotic pathways are presently known in other cell types: (i) direct activation of caspase-8 and (ii) induction of ceramide-mediated mitochondrial activation, both leading to subsequent caspase-3 activation. In the present study, five of 11 melanoma cell populations were shown to release cytochrome *c* from mitochondria, which activates caspase-3 and finally results in DNA fragmentation upon treatment with the agonistic monoclonal antibody CH-11. In contrast, this apoptotic pathway was not activated in the remaining six melanoma cell populations. Interestingly, the susceptibility of melanoma cells to CD95L/FasL-triggered cell death was clearly correlated with *N*-acetylsphingosine-mediated apoptosis. Our results are in line with a defect upstream of mitochondrial cytochrome *c* release in resistant cells.

Key words: CD95/Fas; Signaling; Mitochondrion; Melanoma; Cytochrome *c* release

1. Introduction

APO-1/Fas, now called CD95, was the first member of the tumor necrosis factor (TNF) receptor subfamily named death receptors, described in terms of its function to trigger apoptosis upon binding of its ligand (CD95/Fas Ligand (CD95L/FasL)) or specific agonistic antibodies [1]. Stimulation of CD95 results in oligomerization of the receptors and recruitment of two key signaling proteins, adapter protein-FADD (Fas-associated death domain, also called MORT-1) and caspase-8 [2], forming a death inducing signaling complex (DISC) [3]. Caspase-8 activation is followed by caspase-3 activation which seems to be the most important effector in this apoptotic pathway [4]. Besides the FADD/caspase-8 pathway, a novel CD95-mediated apoptotic pathway with involvement of mitochondria has been identified and its pivotal role as orchestrator of apoptosis has been firmly established [5]. During apoptosis in vitro and in vivo cytochrome *c* is released from mitochondria, and cytosolic cytochrome *c* forms an essential part of the vertebrate 'apoptosome', composed of cytochrome *c*, apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 [6]. This results in activation of caspase-9, which then processes caspase-3, whereby the cell passes the threshold of no return. Cytochrome *c* release can be inhibited by anti-apoptotic members of proteins of the B-cell lymphoma proto-oncogene 2 (bcl-2) family which are described as the effectors of this apoptotic pathway [7]. Another pathway implicated in death receptor-mediated apoptosis involves the generation of ceramide by hydrolysis of the membrane sphingophospholipid sphingomyelin [8]. Ceramide is a signaling molecule involved in cellular responses to a variety of apoptotic stimuli [9]. It is produced upon activation of sphingomyelinases (SMases) or via de novo synthesis by ceramide synthase [10,11].

In experiments overexpressing bcl-2, ceramide-stimulated cells showed a significant decrease of cell death, thereby revealing the importance of mitochondrial function in ceramide-mediated apoptosis [12]. In U-937 cells, ceramide increase was shown after treatment with agonistic anti-Fas antibodies and exogenous addition of cell-permeable *N*-acetylsphingosine (C_2-ceramide) induced apoptosis in various malignant cells [13]. Dysregulation of the Fas-signaling system was shown to contribute to formation and growth of neoplasia including malignant melanoma. Melanoma is a tumor showing an increasing incidence and aggressive biological behavior. Melanoma cells defy endogenous apoptosis and resist very effectively to different physiological mechanisms triggering the apoptotic cascade and causing cell death, such as CD95/CD95L [14]. However, the role of ceramide-mediated apoptosis for the development of CD95/CD95L resistance in melanoma cells has not been investigated yet.

In the present study, we determined the susceptibility of nine melanoma cell lines and two primary cultures from melanoma lesions to apoptosis mediated by CD95 agonistic antibodies and truncated ceramides. We demonstrate here, that the susceptibility to these apoptotic stimuli was in correlation

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Abbreviations: TNF, tumor necrosis factor; CD95L/FasL, CD95/Fas ligand; mAb, monoclonal antibody; C_2-ceramide, *N*-acetylsphingosine; FADD, Fas-associated death domain; Apaf-1, apoptotic protease activating factor-1; bcl-2, B-cell lymphoma proto-oncogene 2; RT-PCR, reverse transcription-polymerase chain reaction; Ac-DEVD, Ac-Asp-Glu-Val-Asp; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DISC, death inducing signaling complex; FLIP, FLICE-inhibitory protein

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with a defect in cytochrome *c* release and the resulting loss of caspase-3 activation.

2. Materials and methods

2.1. Melanoma cells and reagents

Two melanoma cell populations (M186, M221) were obtained from patients with histologically confirmed metastatic melanoma by surgical intervention. Four out of nine established human melanoma cell lines used in this study originated from primary tumors: A-375 [15], Bro [16], JPC-298 [17], Mel-HO [18], and five cell lines originated from metastases: Mel-2A [19], MeWo [20], SK-Mel-13, SK-MEL-23, SK-Mel-28 [21]. All melanoma cell lines were grown in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin. Agonistic anti-CD95 monoclonal antibodies CH-11 (Chemicon, Hofheim, Germany) were used at a final concentration of 1 μg/ml. C_2-ceramide and C_8-ceramide were purchased from Alexis (Gruenberg, Germany) and dissolved in ethanol to give a 10 mM stock solution.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) detection

Total RNA was isolated according to the manufacturer's protocol with a commercially available kit (Quiagen, Hilden, Germany). The RNAs were converted into cDNA with the kit from Gibco BRL (Karslruhe, Germany) according to their instructions. PCR was performed with gene-specific, intron-spanning primers at a final concentration of 1 mM dNTPs, 10 μM each of the primers and 2.5 Units (U) of *Taq* DNA polymerase (Perkin Elmer, Heidelberg, Germany) in a total volume of 50 μl. Thermal cycling was carried out as follows: one cycle of 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min and an additional extension period at 72°C for 5 min. An aliquot of 20 μl of each reaction was separated by electrophoresis using a 2% agarose gel and visualized with ethidium bromide.

The primer pairs were purchased from TibMolbiol (Berlin, Germany). Sequences are available under http://www.medizin.fu-berlin.de/ceramide/protocols/primers.html.

2.3. Cytotoxicity assays

Cytotoxicity was determined with the cytotoxicity detection kit (lactate dehydrogenase (LDH)) from Roche Diagnostics (Mannheim, Germany) exactly as described elsewhere [22]. Extinction values of control cells were set as 100% and the rate of LDH release from treated cells was calculated as % of control.

2.4. Determination of apoptosis

Confluent cells in 24-well plates were treated with the respective agents or control vehicle in DMEM. After 12 h of incubation, cell death was measured in a photometric enzyme-immunoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) with the commercially available kit 'Cell death detection ELISA^{PLUS}' from Roche Diagnostics (Mannheim, Germany), exactly as described elsewhere [22]. Extinction values of control cells were set as 100% and the rate of LDH release from treated cells was calculated as % of control.

2.5. Radiolabelling of cells and sphingomyelin quantitation

Cells were labelled with choline as described previously [23]. Medium was removed and pulse medium (DMEM containing 3.7×10^4 Bq/ml [*methyl*-^{3}H]choline) was added. After incubation for 72 h, cells were washed twice with phosphate-buffered saline (PBS) and then treated with 1 μg/ml CH-11 antibodies in DMEM. After incubation for 8 and 10 h, cells were harvested in 400 μl ice-cold PBS. After freeze-drying of the cells, lipids were extracted as described [24]. Total lipid extracts were dried under a stream of nitrogen and stored at −20°C. Sphingomyelin was quantified using bacterial SMase to release [^{3}H]phosphocholine as described recently [25]. The radioactivity in control samples was set as 100%. Subsequently, sphingomyelin in the samples of CH-11 treated cells was calculated as percent of control.

2.6. Preparation of cytosolic extracts and mitochondria

Mitochondrial and cytosolic extracts were prepared according to a method described previously [26]. After incubation with the respective substances, cells were harvested in PBS, equilibrated in hypotonic buffer (20 mM HEPES (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA) and centrifuged at $300\times g$ for 5 min. The resulting pellet was then dissolved in hypotonic buffer with addition of PMSF (f.c. 0.1 mM) and incubated on ice for 15 min. Cells were homogenized by passing the cells through a syringe (G 20) approximately 20 times. The membranes were isolated by two-fold centrifugation at $10\,000\times g$ at 4°C for 10 min and the supernatant of the second centrifugation was used as cytosolic extract. The resulting mitochondrial pellets were solubilized in lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl containing 1 μM leupeptin, 1 μM pepstatin and 100 μM phenylmethyl sulfonylfluoride) and together with the clear supernatants frozen at −80°C. Western blot analyses with cytosolic and mitochondrial extracts were performed as described below and conducted with mouse anti-cytochrome *c* antibodies (Pharmingen, Hamburg, Germany) with a 1:1000 dilution. The second horseradish peroxidase-coupled anti-mouse antibody (Dako, Hamburg, Germany) was used at a 1:10 000 dilution.

2.7. Determination of caspase-3 processing in intact cells (in vivo)

After treatment of melanoma cells (A375, M186, Mel2A, M221) with 1 μg/ml anti-CD95 antibodies for 6 h or 30 μM C_2-ceramide for 4 h, cells were washed with PBS and lysed with lysis buffer as described above. Then, 45 μg of cytosolic protein were separated on a 15% SDS–polyacrylamide gel electrophoresis (PAGE) and blotted to nitrocellulose membrane as described [27]. Membranes were blocked for 1 h with 3% non-fat dry milk in PBS containing 0.25% Tween-20 and then incubated with rabbit polyclonal antibodies to caspase-3 (Pharmingen, Hamburg, Germany) in 1:2000 dilution. The blots were counterstained with goat anti-rabbit IgG conjugated with horseradish peroxidase (Promega, Mannheim, Germany) in 1:4500 dilution for 1 h. The immunoreactive bands were visualized by incubation of the membrane with enhanced chemiluminescence reagent ECL from Pierce (Rockford, IL, USA). Interblot reproducibility of identical samples was checked and detection of caspase-3, as determined by Western blot and subsequent videodensitometry, showed a coefficient of variation of 8.3%. The detection limit for the 17 kDa cleavage product of caspase-3 which represents the active form of the enzyme was below 0.1 mU (data not shown).

2.8. Determination of caspase-3 and caspase-9 activation in cell-free extracts

To measure caspase activation in vitro, cytosolic extracts of melanoma cells were prepared as described above. To in vitro activate caspase-3 and caspase-9, 10 μM cytochrome *c* (Sigma, Munich, Germany), 1 mM dATP and 1 mM dithiothreitol were added to cytosolic extracts. After incubation for 30 min at 30°C, 10 μl of this enzyme extract, 2 μl caspase-3 substrate Ac-Asp Glu-Val-Asp-pNA (Ac-DEVD-pNA; Calbiochem, Bad Soden, Germany) or caspase-9-substrate Ac-LEHD-pNA (Chemicon, Hofheim, Germany) and 90 μl of buffer B (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS and 10% saccharose) were incubated for up to 90 min at 37°C. Photometrical measurements were performed at 405 nm in an ELISA-reader. As negative controls, cellular extracts were incubated in the absence of cytochrome *c* and dATP, and caspase-3 and caspase-9 activities were determined as described above. One unit of enzyme activity is defined as μmol Ac-DEVD-pNA or μmol Ac-LEHD-pNA cleaved per min.

3. Results

3.1. Susceptibility of melanoma cells to CD95-mediated apoptosis by CH-11 monoclonal antibody (mAb)

In preliminary experiments, 1 μg/ml agonistic anti-CD95 mAb CH-11 was shown to be the most effective concentration for apoptosis induction (data not shown). A panel of melanoma cell cultures was treated for 12 h with CH-11 and apoptosis was determined morphologically by observation under light microscope and measured photometrically using an enzyme immunoassay for determination of cytoplasmic histone-associated DNA fragments. As listed in Table 1, CH-11-in-

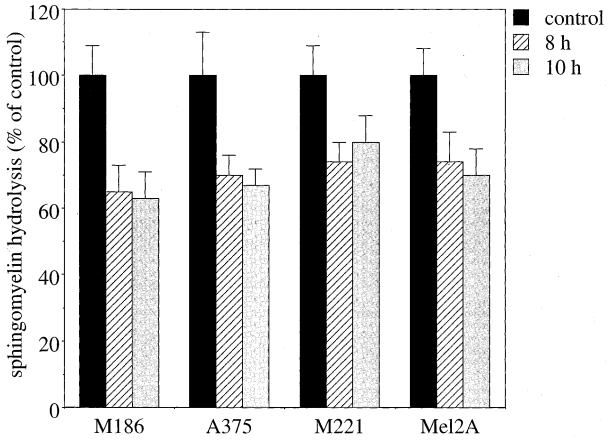

Fig. 1. CH-11 induces sphingomyelin hydrolysis in both sensitive and resistant melanoma cells. Sensitive (A375, M186) and resistant (Mel-2A, M221) melanoma cells which had been previously labelled for 72 h with [^{3}H]choline were washed with PBS and stimulated with CH-11 for 8 and 10 h. Subsequently, cells were washed with ice-cold PBS and harvested in PBS. Cellular lipids were extracted according to Bligh and Dyer and sphingomyelin hydrolysis was determined as described in Section 2. Values are given as percent of control ± S.D. ($n = 3$).

duced apoptosis occurred in four melanoma cell lines (MelHo, Bro, SKMel 13 and A375) and one primary culture (M186). In contrast, JPC298 cells, MEWO cells, SkMel28 cells, SkMel23 cells and Mel2A cells remained completely resistant to CH-11 stimulation (Table 1) even after prolonged incubation times of 36 h (data not shown).

3.2. Determination of the effectors of the CD95 signaling pathway in different melanoma cells

In order to investigate the role of key effectors in the CD95 signaling pathway, we screened the expression of CD95-associated genes with RT-PCR in the panel of nine melanoma cell lines and two primary cultures from melanoma lesions. To test the relative quantity of cDNAs, each of them was screened for the housekeeping gene Ribo S9 and for the absence of genomic DNA contamination. For all screened genes intron spanning primers were used. The colon adenocarcinoma cell line, SW 480, expressing CD95L mRNA [28] was taken as a positive control. None of the 11 melanoma cell lines expressed CD95L mRNA and this result was confirmed with Northern blot analysis, where no expression of CD95L-

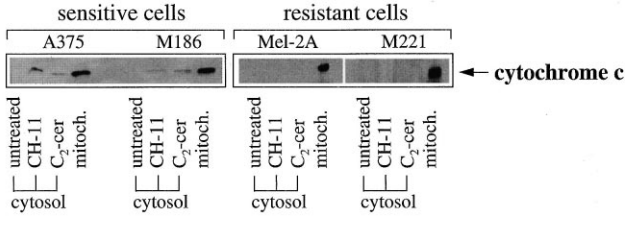

Fig. 2. Cytochrome *c* is released upon stimulation with CH-11 and C_2-ceramide in sensitive melanoma cells. For the detection of cytochrome *c* release, cells were harvested after stimulation with CH-11 for 6 h and C_2-ceramide for 4 h and cytosolic and mitochondrial extracts were prepared according to the protocol mentioned in Section 2. Western blot was performed with anti-human cytochrome *c* antibodies. The experiment was repeated twice and similar results were obtained.

Table 1
Susceptibility of melanoma cell populations to CH-11 mAb and C_2-ceramide

Cell populations	CH-11	C_2-ceramide
JPC298	−	−
MEWO	−	−
A375	+++	+++
SkMel28	−	−
BRO	+	+
M186	++	++
M221	−	−
MelHo	++	++
SkMel23	−	−
SkMel13	+	+
Mel2A	−	−

The panel of melanoma cell populations was treated for 12 h with 1 μg/ml of agonistic anti-CD95 CH-11 mAb. The same cell populations were treated with exogenously applied 30 μM cell-permeable C_2-ceramide for 6 h. Apoptosis was measured photometrically using an enzyme immunoassay for determination of cytoplasmic histone-associated DNA fragments (for details, see Section 2). Results of apoptosis detection: −, resistant; +, weak; ++, intermediate; +++, strong sensitivity.

mRNA could be detected (data not shown). On the other hand, significant expression levels of CD95, adapter protein FADD, caspase-8 and caspase-3 mRNA were found in resistant as well as sensitive melanoma cells.

In another set of RT-PCR experiments, we compared the gene expression of bcl-2, bcl-x_L, bax and FLIP (FLICE-inhibitory protein) in resistant and sensitive melanoma cell lines, but no differences in gene expression on mRNA level could be detected.

3.3. Induction of sphingomyelin hydrolysis by activation of the CD95 signaling pathway in melanoma cells

CD95 ligand induces apoptosis via activation of the sphingomyelin cycle in U937 cells [29]. Triggering of the CD95 cell-surface receptor with 1 μg/ml CH-11 induced hydrolysis of approximately 30% of the total sphingomyelin in both sensitive and resistant melanoma cells (Fig. 1). These results ex-

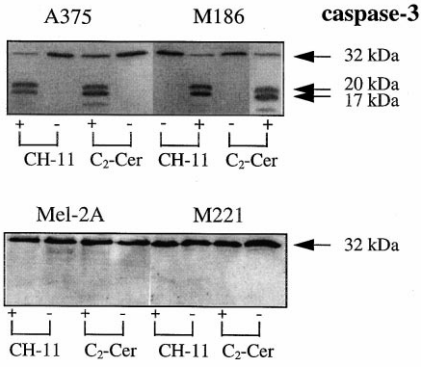

Fig. 3. Activation of caspase-3 after stimulation with CH-11 and C_2-ceramide in sensitive (A375, M185) and resistant (SkMel23, M221) cells. Cells were stimulated with 1 μg/ml CH-11 mAb and C_2-ceramide for 6 and 4 h, respectively. Then, protein extracts were prepared and separated by SDS–PAGE. Western blot was carried out as described in Section 2. Arrows at the right margin indicate the migration positions of procaspase-3 (32 kDa) and its cleavage products (17 and 20 kDa) recognized by the anti-human caspase-3 Ab used in the immunoblot. The experiment was repeated twice and similar results were obtained.

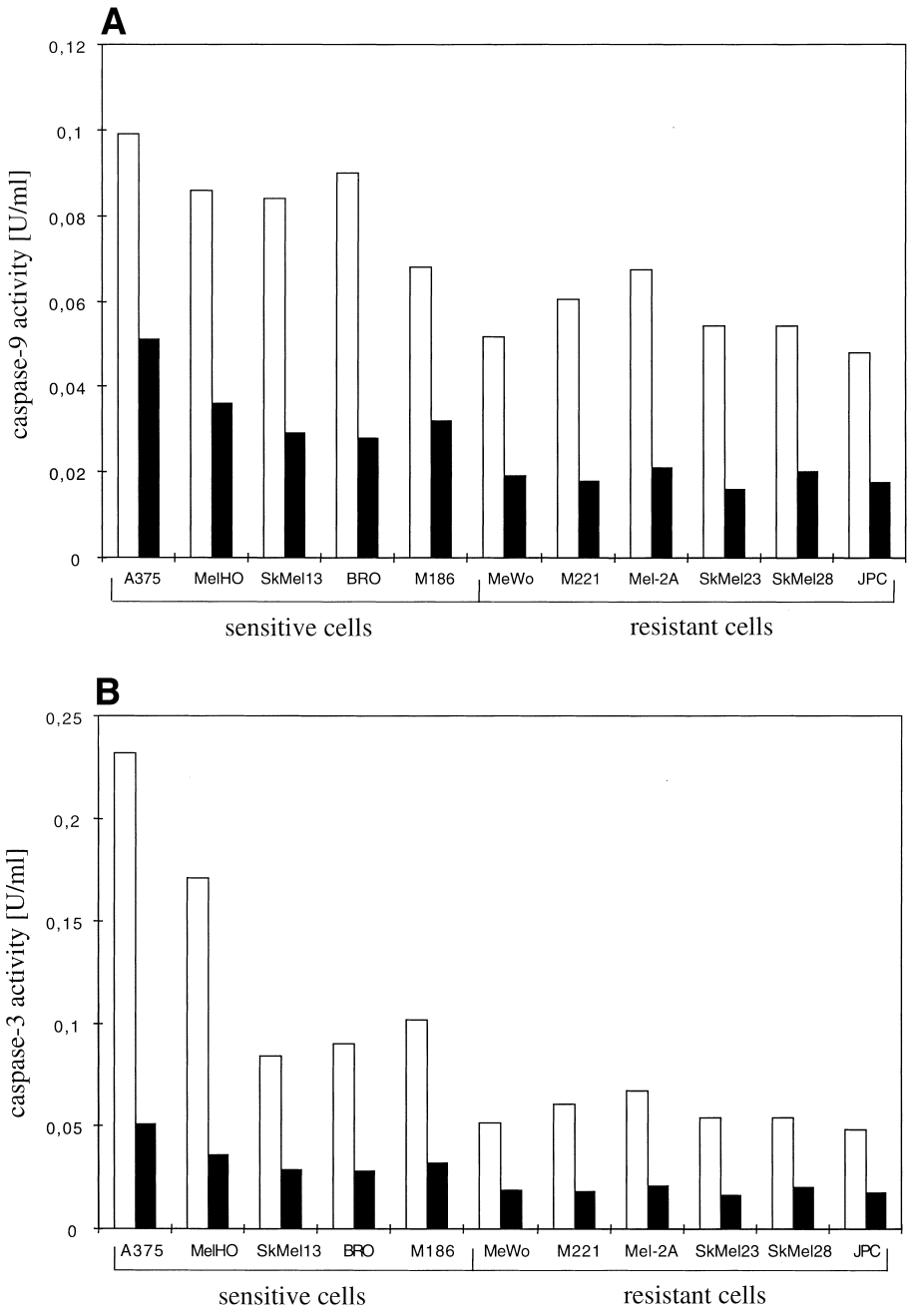

Fig. 4. Caspase activation in vitro. Cytosolic extracts were incubated with 10 μM cytochrome *c*, 1 mM dATP and 1 mM dithiothreitol for 30 min in hypotonic buffer as described in Section 2. Then, caspase-9 (A) and caspase-3 (B) activity were measured at 37°C using Ac-LEHD-pNA and Ac-DEVD pNA, respectively. Photometrical measurements were done at 405 nm in an ELISA-reader. As negative controls, cell extracts which were likewise incubated in the absence of cytochrome *c* and dATP were used. The caspase enzyme activities were determined as maximum initial velocity V_{max}. Data represent the mean of two determinations and are given as U/μg protein. The experiment was repeated twice and similar results were obtained.

clude the possibility of a defect at the level of SMase in resistant cells.

3.4. Susceptibility of melanoma cells to C_2-ceramide

In order to investigate the possible role of ceramide in our system, melanoma cell lines were treated with 30 μM C_2-ceramide for 6 h. Unspecific cytotoxic effects of C_2-ceramide on melanoma cells were determined after 2 h of incubation by measurement of the release of LDH activity into cell culture supernatants. It was shown that 30 μM C_2-ceramide did not cause necrotic cell death in melanoma cells (data not shown). On the other hand, C_2-ceramide was able to induce apoptosis in four melanoma cell lines (MelHo, Bro, SKMel 13 and A375) and one primary culture (M186) with different sensitivity among these cells (Table 1). The other melanoma populations tested (JPC298, MEWO, SkMel28, SkMel23, Mel2A) remained completely resistant to 30 μM C_2-ceramide (Table 1) even after longer incubation periods up to 36 h (data not shown). The same results were obtained with C_8-ceramide (data not shown). Thus, our observations might be also rep-

resentative for long chain ceramides, as has been reasoned recently [30]. As listed in Table 1, there is a complete correlation between the sensitivity of melanoma cells towards C_2-ceramide and their susceptibility towards CH-11 treatment. This was found to be highly significant ($P = 0.0022$, Fisher's exact test).

3.5. Cytochrome c release occurs only in CD95/C_2-ceramide sensitive cells

It has been shown recently that ceramide acts upstream of mitochondria and induces cytochrome *c* release from these organelles [31]. Thus, cytosolic extracts of melanoma cells were prepared and cytochrome *c* release after stimulation was determined by Western blot analysis. Mitochondrial extracts of the different cell lines were included as positive controls. As shown in Fig. 2, stimulation of the sensitive melanoma cells A375 and M186 with CH-11 and C_2-ceramide provoked the translocation of cytochrome *c* into the cytosol. In contrast, in the resistant cells Mel-2A and M221 cytochrome *c* release was absent after 6 h of incubation with CH-11 and after 4 h of incubation with C_2-ceramide. Even after treatment with CH-11 for 8 and 10 h or C_2-ceramide for 6 and 8 h, cytochrome *c* was not detectable in cytosolic extracts of resistant cells (data not shown).

3.6. Activation of caspase-3 in melanoma cells after stimulation with anti-CD95 mAb and C_2-ceramide

To further analyze the downstream mitochondrial pathway in both cell types, caspase-3 processing and activation was analyzed by Western blot using anti-caspase-3 antibody which detects the proenzyme and cleaved forms of the enzyme. After 6 h of treatment with 1 μg/ml CH-11 we detected the active 17 kDa cleavage form of caspase-3 in the sensitive cells A375 and M186 (Fig. 3). In accordance with their failure to release cytochrome *c*, the resistant cells Mel-2A and M221 did not show any cleavage product of caspase-3 indicating that caspase-3 was not active. Similar experiments were carried out after C_2-ceramide treatment for 4 h. The two sensitive melanoma cell populations showed the active cleavage form, while the two resistant populations did not show any caspase-3 activation (Fig. 3) even after 8, 10 and 12 h of treatment (data not shown).

In order to exclude a defect in the process of caspase-activation in resistant melanoma cells, cytochrome *c* and dATP were directly added to cytosolic extracts of the respective cell line. After incubation for 1 h at 30°C, caspase-3 and caspase-9 activities were measured in a colorimetric assay. All CD95- and C_2-ceramide sensitive (A375, Mel-HO, Bro, M186, SK-Mel-13) as well as the resistant melanoma cells (SK-Mel-23, SK-Mel-28, M221, JPC, MeWo, Mel-2A) showed activation of caspase-9 (Fig. 4A) and caspase-3 (Fig. 4B).

4. Discussion

Melanoma cells have been shown to defy CD95-mediated apoptosis in vitro, despite the CD95 expression [14]. This fact suggests a possible correlation with the situation in vivo, where CD95-positive melanoma cells escape the immune response of the CD95L-positive tumor infiltrating T-lymphocytes.

In the present study, we tried to elucidate the mechanisms underlying the resistance to CD95-mediated apoptosis in human melanoma cells. For this, nine melanoma cell lines and two primary melanoma cultures from melanoma lesions were tested for their susceptibility to CD95-mediated apoptosis. We found that four melanoma cell cultures and one primary culture were CD95 sensitive and six melanoma cell lines including one primary culture were resistant. It has recently been shown that the CD95 expression level does not correlate with the sensitivity to CD95-induced apoptosis [32]. Furthermore, CD95 mutation in melanoma is a rare event occurring in only 6.8% of primary cultures from melanoma patients and should be of minor importance for development of CD95 resistance [33]. Therefore, we focussed our interest on death signaling downstream of the death receptor. We reasoned that the activation state and resistance to apoptosis might be attributed to absence of key effectors in CD95 signaling pathways. However, there was no difference in gene expression pattern including effectors, inhibitors and regulators of CD95-induced cell death in any of the cell populations determined by RT-PCR. The findings that the same panel of CD95 sensitive melanoma cells was also susceptible to truncated ceramides whereas the remaining melanoma cell populations were resistant for both stimuli led us to search for the blockade in the joint section of CD95/C_2-ceramide apoptotic pathways, downstream of FADD.

Only the stimulation of sensitive melanoma cells in vivo with CH-11 released cytochrome *c* into the cytosol and led to activation of caspase-3, while resistant melanoma cells failed to translocate cytochrome *c* and to activate the downstream execution process. This points out the central role of mitochondria as key regulators in susceptibility to CD95L and C_2-ceramide in melanoma cells and reveals that the resistance of melanoma cells to apoptotic stimuli such as CD95L and C_2-ceramide is caused by a dysregulation of mitochondrial executional functions. The key regulators of cytochrome *c* release from mitochondria are represented by different members of the bcl-2 protein family. Some of the members of the bcl-2 family, e.g. bad and bax, are promoters, whereas others, e.g. bcl-2 and bcl-x_L, are inhibitors of apoptosis [34]. It seems that the release of cytochrome *c* and other apoptogenic proteins is determined by the relative amounts of apoptosis-promoting and apoptosis-inhibiting bcl-2 proteins in the outer membrane of the mitochondrion. Inappropriate overexpression of bcl-2 plays a role in promoting various types of tumors, e.g. human follicular B cell lymphoma, and disbalance in the bcl-2/bax ratio has been shown to be a determining factor for breast cancer [35–37]. However, bcl-2 expression is a common finding in melanocytic lesions regardless of their biological behavior and the progression of malignant melanoma does not depend on the high expression of bcl-2 [38,39].

A discussion about the role of ceramide in the CD95 signaling pathway has been raised [40,41] and it is still a matter of debate whether ceramide is functionally involved in the propagation of CD95 death signal or just represents a secondary modulatory pathway. In this context our results, showing that increasing intracellular ceramide levels via exogenous addition of truncated ceramides specifically induced apoptosis in CD95 sensitive cells, support the significant role of ceramide in the CD95 apoptotic pathway in human melanoma cells.

The above discussed cell line data are corroborated by the results obtained from the sensitive and resistant primary cultures. Thus, the different susceptibility of melanoma populations in vitro may reflect the situation in vivo, where various

malignant melanomas show differences in their aggressive biological behavior. Furthermore, malignant melanomas also show diverse sensitivity to apoptotic stimuli, e.g. to CD95L expressed on tumor-infiltrating lymphocytes, γ-irradiation or chemo- and immunotherapeutics. Taken together, our data demonstrate for the first time that this diversity may be related to their potential to release cytochrome *c* from mitochondria in these cells.

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