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Impaired activation of caspases and prevention of mitochondrial dysfunction in the metastatic colon carcinoma CC531s-m2 cell line

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Abstract

In a previous paper we described the properties of a rapidly metastasizing cell line CC531s-m2 derived from the poorly metastasizing CC531s cell. The m2-cell line was relatively resistant to killing by NK cells. Both CD95L and TRAIL mediated apoptosis was decreased in the m2-cell line. Now, by flow cytometrical analysis of intra- and extra-cellular expressed receptors, we show that the localization of the receptors for CD95L and TRAIL was not altered in the CC531s-m2 cells as compared to the parental cell line. Subsequently caspase-activation and mitochondrial function were studied by enzymatic cleavage of fluorescent caspase-substrates and retention of the mitochondrial dye rhodamine-123, respectively. The activation of caspases as well as the loss of the mitochondrial membrane potential (MMP) was less in the CC531s-m2 cell line upon CD95L- and TRAIL-signalling. Furthermore, the sensitivity of the CC531-m2 towards cisplatin-induced apoptosis was strongly decreased. This was consistent with less mitochondrial damage, delayed caspase cleavage and decreased caspase activity. Altogether, we conclude that an Natural Killer-cell insensitive cell is less sensitive to CD95L- and TRAIL-induced apoptosis as well as anti-cancer drug induced apoptosis by prevention of mitochondrial damage and activation of caspases.

Keywords: CD95L; TRAIL; Apoptosis; Caspase activity; Cisplatin; Metastasizing cell line

1. Introduction

Metastases arise from cells that detach from the primary tumor, migrate into blood or lymph vessels, survive in the vasculature and extravasate again from the circulation [1]. During this process, the cells need to escape immunosurveillance. Two types of cytotoxic lymphocytes are involved in killing of circulating tumor cells: the T-lymphocyte and the Natural Killer-cell. Thus, cells that have protected themselves somehow from being lysed by these lymphocytes have a higher change to form metastases.

Both T- as well as Natural Killer-cells use two strategies to kill their targets: the death receptor mediated pathway and the exocytosis pathway. The first strategy comprises expression of members of the tumor necrosis factor (TNF)-super family (such as CD95L, TRAIL [2]) on the outer membrane of the lymphocytes that interact with receptors on the outer membrane of the target cell. CD95L (also known as FasL or Apo1-L) will bind to CD95 [3], the only membrane-expressed receptor for CD95L. TRAIL can bind to two death receptors, DR4 and DR5, and two decoy receptors, DcR1 and DcR2 [4]. Binding of either ligand to a death receptor leads to formation of the death-inducing signaling complex (DISC). Subsequently, via the adaptor molecule FADD, caspase-8 is recruited and activated [5], which can either directly or indirectly activate the downstream caspase-3, leading to apoptosis. In the direct route, caspase-8 directly cleaves pro-caspase-3 into the active form, causing the onset of apoptosis. In the indirect route, caspase-8 cleaves pro-apoptotic members of the bcl-2 family that will translocate to the mitochondrial membrane, causing depolarization [6,7]. Subsequently, cytochrome c is released that forms a complex with APAF-1, ATP and pro-caspase-9 called the apoptosome

Abbreviations: CD95L, CD95ligand; CDDP, *cis*-diamminedichloroplatinum; DcR, decoy receptor; DR, death receptor; TRAIL, tumor necrosis factor related apoptosis inducing ligand

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[8]. Caspase-9 is then activated within this complex and will cleave the effector caspase-3, resulting in apoptosis.

The second strategy, the exocytosis pathway, comprises the secretion of the pore-forming protein perforin and the serine protease granzyme B [9,10]: perforin enables granzyme B to enter the cytoplasm of the target cell and granzyme B can cleave and thereby activate several substrates amongst which caspases, a family of proteases [11]. These caspases are the executioners of apoptosis that regulate cell death.

CDDP is used to treat tumors of various origins [12]. Although widely used, its exact molecular mechanism of killing is not yet clear. Park et al. [13] however, nicely showed that cisplatin causes cell death by acting directly on the mitochondria and subsequently activating the caspase-routes. In addition, we previously showed that the nephrotoxic effects of CDDP could also be attributed to mitochondrial damage [14,15].

Previously [16], we reported selection and isolation of tumor cells (CC531s-m2 cells) that rapidly form metastases in vivo. Because the process of metastasis requires escape from immunosurveillance, we investigated the susceptibility of the cell lines towards apoptosis. These cells were less sensitive to CD95L- and TRAIL-mediated apoptosis as compared to the parental cells, CC531s. In this paper, we investigated the molecular mechanisms involved in the decreased sensitivity of CC531s-m2 cells to CD95L and TRAIL induced apoptosis. We already reported using western blotting that the decreased response to both CD95L and TRAIL was not due to a decreased expression of the receptors for both ligands. Therefore, we now report on receptor localisation and down-stream signalling in the CC531s-celll lines exposed to TRAIL and CD95L using CDDP as a control. We conclude that the cause of the relative insensitivity of CC531s-m2 cells to death-signals is a result of decreased activation of caspases at the level of the mitochondria.

2. Materials and methods

2.1. Cell lines

All CC531s-cells (developed by Marquet et al. [17] using the carcinogen dimethylhydrazin in Wag/Rij rats) were cultured in RPMI 1640 'Dutch modification' (Gibco) supplemented with 10% heat-inactivated FCS, 100 μg/ml streptomycin, 100 IU/ml penicillin and 4 mM L-glutamine (all Gibco).

Mouse B cell lymphoma 2PK3-mock, 2PK3-mCD95L and 2PK3-mTRAIL cells (a gift from Dr. Hideo Yagita, Tokyo, Japan [18]) were cultured in RPMI 1640 (L-glutamine, 25 mM HEPES) supplemented with 10% heat-inactivated FCS, 100 μg/ml streptomycin and 100 IU/ml penicillin (Gibco). Selection of transfected cells was achieved by addition of 100 μg/ml G-418 (Gibco).

2.2. Reagents and antibodies

The rabbit anti-CD95 (FL335) and anti-DR4 (H-130) antibody was purchased from Santa Cruz Biotechnologies, the rabbit anti-DR5 from Stressgen Biotechnologies and the rabbit anti-DcR1 and anti-DcR2 from Prosci. The alexa488-conjugated secondary antibody and rhodamine-123 was obtained from Molecular Probes. Srinivasan [19] kindly provided the antibody against active caspase-3.

2.3. Receptor expression

The receptor expression assay was adapted from Kashii et al. [20]. CC531s cells were harvested by trypsinization and fixed in 4% aq. formaldehyde for 15 min at room temperature. After fixation, the samples were split into two fractions: one fraction for the assay of membrane-bound- and one for that of total receptor expression. The first fraction was resuspended in PBS-EDTA containing 0.5% BSA, the second fraction in the same buffer supplemented with 0.1% triton X-100 and both were incubated for 15 min at 4 °C while shaking continuously. Next, the cells were spun down, washed twice with PBS-EDTA and incubated for 15 min in PBS-EDTA containing 2% FBS. The cells were spun down, resuspended in primary antibody solution (1:200) in PBS-EDTA-BSA and incubated at 4 °C for 45 min while shaking continuously. The cells were washed twice in PBS-EDTA-BSA and incubated with a goat-anti-rabbit-alexa488 secondary antibody for 30 min at 4 °C while shaking. Again, the samples were washed twice and analyzed on the FACScalibur (BD, San Jose, CA, USA). Shortly before the measurement, propidium-iodine was added to label permeabilized cells.

2.4. Mitochondrial membrane potential measurements

On day 1, 1×10^6 CC531s and CC531s-m2 cells were plated in a well of a 6-well plate (Greiner). The next day, the cells were loaded with 1 μM rhodamine-123 for 15 min at 37 °C. The cells were washed twice with culture medium. Next, 2PK3-mock, 2PK3-mCD95L or 2PK3-mTRAIL cells were added at an effector:target-ratio (E/T) of 10 for 0, 90 or 180 min. After the incubation-period at 37 °C, the 2PK3 cells were washed away by briefly incubating the cells with 0.2% EDTA (w/v) in PBS, while gently tapping. The CC531s cells were harvested by trypsinization and analyzed on the FACScalibur.

2.5. Caspase-activity

On day 1, 1.5×10^6 CC531s and CC531s-m2 cells were plated in a well of a 6-wells plate (Greiner). The next day, 2PK3-mock, 2PK3-mCD95L or 2PK3-mTRAIL cells were added at an E/T-ratio of 10 for 0, 60, 120 and 180 min. After the incubation-period at 37 °C, the 2PK3 cells were removed by briefly incubating the cells with 0.2% ETDA

(w/v) in PBS, while gently tapping. The CC531s cells were harvested by trypsinization. When cells had detached, culture medium was added. The cells were spun down in a 15-ml tube (Greiner) and washed with PBS. The pellet was resuspended in lysis-buffer (consisting of 10 mM HEPES, 40 mM glycerophosphate, 50 mM NaCl, 2 mM MgCl2 and 5 mM EGTA, pH 7.0) en cells were transferred to an Eppendorf 1.5 ml reaction tube. The caspase-activity was determined as described previously [21] using Ac-DECD-AMC as a caspase-3 substrate, Ac-IEPD-AMC as a caspase-8 substrate and Ac-LEHD-AMC as a caspase-9 substrate (all Bachem, Bubendorf, Switzerland). Upon cleavage of a substrate by the active caspase AMC is released which results in a fluorescent signal. Using standard concentrations of purified AMC, the caspase-activity was set out as the concentration of AMC (in pmol) that was cleaved in 1 min by 1 mg of protein.

2.6. Immunoblotting

The CC531s and CC531s-m2 cells were exposed to CD95L- and TRAIL expressing cells similar as in the caspase-activity assay. Next, the CC531 cells were scraped and lysed in TSE buffer (10 mM Tris–HCl, 250 mM sucrose, 1 mM EGTA, pH 7.4, containing 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate, 50 mM sodium fluoride and 1 mM phenylmethylsulfonyl fluoride). The protein concentration in the supernatant was determined using the BioRad protein assay with IgG as a standard. Fifteen microgram of total cellular protein was separated by SDS–PAGE and transferred to PVDF membrane (Millipore). Blots were blocked with 5% (w/v) BSA in TBS-T (0.5 M NaCl, 20 mM Tris–HCl, 0.05% (v/v) Tween-20; pH 7.4) and probed with the primary antibody. Next, blots were incubated with secondary antibody containing HRP and visualized with ECL reagent. (Amersham Pharmacia Biotech). The SDS–PAGE gels were stained with Coomassie blue to check that an equal amount of protein was present in the samples.

2.7. Statistical analysis

Statistical differences between two means were determined using Student's two-sample, equal variance, two-tailed *t*-test. *P*-values lower than 0.05 were considered significant.

3. Results

3.1. Sensitivity to CD95L and TRAIL and expression of their receptors

Previously, we described the selection of a rat colon carcinoma cell line (CC531s-m2) that survived systemic immunosurveillance [16]. Characterization of the CC531s-m2 cell line showed a striking decrease in sensitivity to CD95L- and TRAIL-mediated apoptosis: 46% and 37%, respectively as compared to the parental CC531s cell line which response as set at 100%. Immunoblotting of the CC531s and CC531s-m2 cells for CD95 and the TRAIL-receptors already suggested no alterations in the expression of the receptors at the protein levels [16]. However, the diminished response to CD95L and TRAIL can still be explained by a decreased extra-cellular orientation of the receptors and the concordant increased intracellular expression. Although no mutations are known to us that can cause loss of translocation of all CD95- and TRAIL-receptors, we wanted to exclude this possibility. Therefore, we examined the extra- and intracellular expression of the receptors by flow cytometry. After fixation, the level of receptors present on the outer membrane (non-permeabilized cells) and total receptor levels (permeabilized cells) were determined by flow cytometry; as a reference, the level of receptors present in permeabilized CC531s cells was set as 100%. As shown in Fig. 1, no differences were found in the expression of the receptors between the two cell types. Neither the total amount of receptors nor the membrane-expressed portion of the receptors was altered in CC531s-m2 cells compared to the parental CC531s cells; some 30–45% of the total amount of the receptors was expressed on the outer membrane. Therefore, a down-regulation of the three death receptors or up-regulation of the two decoy-receptors could not explain the decreased response to CD95L and TRAIL.

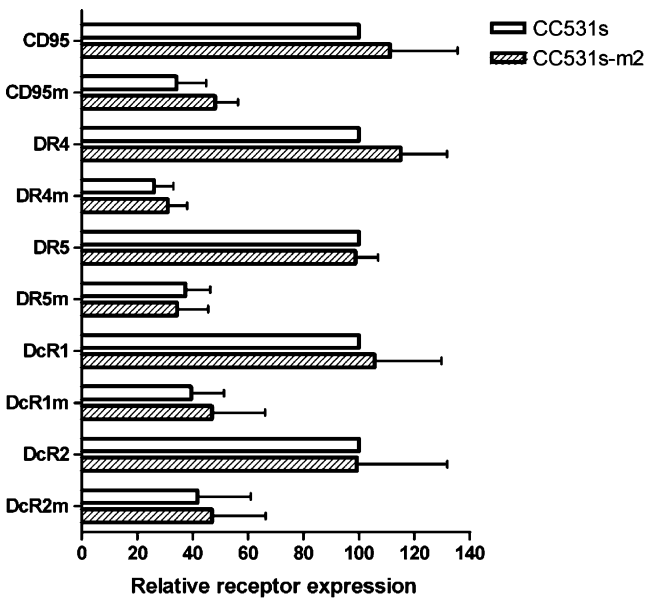

Fig. 1. Receptor expression on CC531s and CC531s-m2 cells. CC531s and -m2 cells were harvested and fixated. Half of each sample was permeabilized to enable intra-cellular staining. Next, the cells were stained with antibodies against the receptors, washed and subsequently incubated with a secondary antibody. The relative total receptor level and the membrane bound receptor level (m) were determined by flow cytometry. The total receptor level in CC531s cells was set as 100%. No statistical differences could be found. Mean values of three independent experiments are shown.

3.2. Caspase-activity

Upon triggering of CD95, DR4 or DR5, the up-stream caspase-8 is activated, that subsequently can directly activate the down-stream caspase-3 or indirectly, via mitochondrial dysfunction, caspase-9 [22]. Therefore, the activity of these caspases in CC531s and CC531s-m2 cells upon CD95L- and TRAIL exposure was compared. CC531s and CC531s-m2 cells were seeded in 6-well plates, exposed to 2PK3-effector cells (expressing CD95L or TRAIL) for the indicated periods, harvested, lysed and the caspase activity was measured. Firstly, the activity of caspase-3, the executioner caspase, was determined (Fig. 2). The caspase-activity after exposure to 2PK3-mCD95L cells (A) as well as to 2PK3-mTRAIL cells (B) was much less in CC531s-m2 cells than in CC531s cells. Differences in caspase-3 activity after CD95L-signaling were seen after 120 and 180 min of co-incubation: at 120 min the caspase-3 activity was 87 pmol AMC/min mg in the CC531s-m2 cells and 115 pmol AMC/min mg in the CC531s cells. One hour later, the maximum caspase-3 activity was detected in the CC531s cells (170 pmol AMC/min mg), whereas two-third of that activity was seen in the CC531s-m2 cell line: 108 pmol AMC/min mg. Upon TRAIL-signaling, the same pattern was observed: differences were detected after 120 and 180 min: at 120 min 57 pmol AMC/min mg caspase-activity was seen in the CC531s-m2 cell line versus 105 pmol AMC/min mg in the parental cell line; at180 min 89 pmol AMC/min mg was measured in CC531s-m2 cell line, whereas in the CC531s cell, the maximum activity was measured: 133 pmol AMC/min mg.

To examine whether differences in caspase-activity could already be detected in the activation of the up-stream caspase-8, the activity of that caspase was determined by cleavage of IEPD-AMC. After 120 min of co-incubation with CD95L-expressing effector cells, 48 pmol AMC/min mg caspase-8 activity was seen in the CC531s cells, against 24 pmol AMC/min mg in the C531s-m2 cell line (Fig. 3A). Sixty minutes later, the activities were 80 pmol AMC/min mg caspase-8-activity in CC531s cells, whereas only 48 pmol AMC/min mg activity could be detected in CC531s-m2 cells. A similar pattern was seen upon TRAIL-exposure: at 120 min 43 pmol AMC/min mg caspase-8 activity was seen in the CC531s cells (Fig. 3B) versus 23 pmol AMC/min mg in the CC531s-m2 cell line. This increased up to 50 pmol AMC/min mg after 180 min in the CC531s-m2 cell line and 72 pmol AMC/min mg in the CC531s cells.

Finally, also caspase-9 activity was determined. Since this caspase becomes activated upon the formation of the

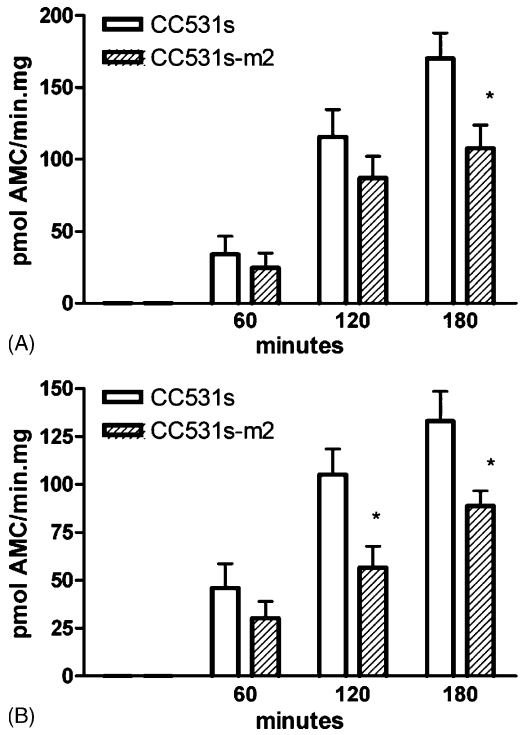

Fig. 2. Caspase-3 activation in CC531s and -m2 cells upon CD95L- and TRAIL-signaling. CC531s and -m2 cells were seeded in 6-wells plates and co-incubated with CD95L- (A) and TRAIL-expressing effector cells (B) for the indicated time-periods. The effector cells were removed, the target cells harvested, lysed and the caspase-activity was determined by quantification of Ac-DEVD-AMC cleavage. Statistically significant differences are marked with an asterisk. Mean values of three independent experiments are shown.

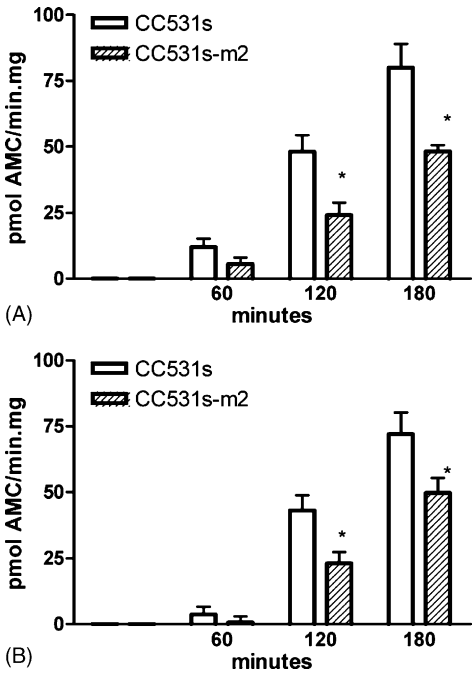

Fig. 3. Caspase-8 activation in CC531s and -m2 cells upon CD95L- and TRAIL-signaling. CC531s and -m2 cells were seeded in 6-wells plates and co-incubated with CD95L- (A) and TRAIL-expressing effector cells (B) for the indicated time-periods. The effector cells were removed, the target cells harvested, lysed and the caspase-activity was determined by quantification of Ac-IEPD-AMC cleavage. Statistically significant differences are marked with an asterisk. Mean values of three independent experiments are shown.

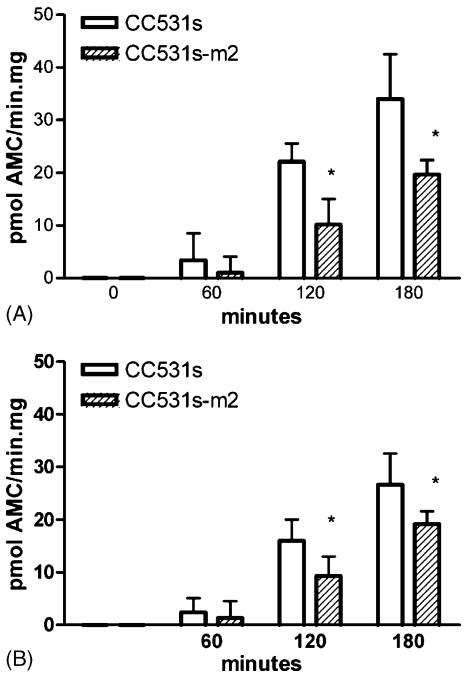

Fig. 4. Caspase-9 activation in CC531s and -m2 cells upon CD95L- and TRAIL-signaling. CC531s and -m2 cells were seeded in 6 wells plates and co-incubated with CD95L- (A) and TRAIL-expressing effector cells (B) for the indicated time-periods. The effector cells were removed, the target cells harvested, lysed and the caspase-activity was determined by quantification of Ac-LEHD-AMC cleavage. Statistically significant differences are marked with an asterisk. Mean values of three independent experiments are shown.

apoptosome, this activity is indicative of the mitochondrial dysfunction caused by both ligands. Again, differences were seen after 120 min of co-incubation with the CD95L-expressing effector cells: 22 pmol AMC/min mg caspase-9-activity was seen in the parental cell line, against 10 pmol AMC/min mg in the CC531s-m2 cells (Fig. 4A). After 180 min, the activity in the CC531s-m2 cells had increased up to 20 pmol AMC/min mg versus 34 pmol AMC/min mg in the parental CC531s cell line. Co-incubation of CC531s cells with 2PK3-mTRAIL cells for 120 min caused 16 pmol AMC/min mg caspase-9 activity (Fig. 4B). The activity in the CC531s-m2 cells was much lower at 120 min (9 pmol AMC/min mg). After 3 h, the decrease in activity was even more pronounced (27 pmol versus 19 pmol AMC/min mg).

3.3. Mitochondrial membrane potential

Differences in activity of all caspases could be detected, including caspase-9 that is activated in the apoptosome. This complex is formed upon the release of cytochrome c from the mitochondria [11]. In order for cytochrome c to be released from the mitochondria, the mitochondrial membrane potential (MMP) has to collapse. The MMP can be assessed by loading the mitochondria with the fluorescent dye rhodamine-123 that is retained in fully functional mitochondria and released from mitochondria that have lost their potential. We loaded CC531s and CC531s-m2 cells with rhodamine-123 and exposed them to 2PK3-mCD95L and 2PK3-mTRAIL cells for 90 and 180 min. After the co-incubation-period, the CC531-cells were flow cytometrically examined for rhodamine-123 fluorescence. After 90 min the MMP had decreased in both cell lines after co-incubation with 2PK3-mCD95L cells, but more in the CC531s cell line as compared to the CC531s-m2 cell line (Fig. 5A). After 180 min, the MMP had decreased even further, but again the MMP had dropped more in the parental cells than in the CC531s-m2 cells (median fluorescence intensity of 80 ± 14 in the CC531s cells versus 200 ± 31 in the CC531s-m2-cell line). A similar pattern was seen after TRAIL-signaling (Fig. 5B): after 90 min the MMP was decreased in both cell lines but the decrease was more pronounced in the CC531s cell line than in the CC531s-m2 cell line. Again, after 180 min more rhodamine-123 was lost from the mitochondria of both cell types, but once again more from the parental cells as compared to the rapidly metastasizing type (median fluorescence intensity of 95 ± 28 in the CC531s cells versus 200 ± 37 in the CC531s-m2 cell line).

3.4. Cisplatin-induced apoptosis

To check whether the diminished activation of caspases was specific for CD95L- and TRAIL-signaling, the unrelated apoptosis inducer cisplatin was used. Cells were exposed to different concentrations of CDDP for 17 h. As shown in Fig. 6, exposure of CC531s cells to 50 μM CDDP caused apoptosis in 43% of the cells. In contrast, the same concentration of CDDP caused apoptosis in only 21% of the CC531s-m2 cells. When the concentration of CDDP was increased to 100 μM, the difference between the two cell lines was less profound (52% versus 40%) but still the parental cell line was more sensitive. To confirm that the processes observed were caspase-dependent, we co-incubated the cells with CDDP and the pan-caspase-inhibitor zVAD-fmk. As shown in Fig. 6, addition of zVAD prevented the appearance of phosphatidylserine on the outer membrane of both cell lines. When apoptosis was assessed by cell cycle measurement (the sub G1/G0 population was regarded as apoptotic), the same patterns in sensitivity of CC531s and -m2 cells were seen (data not shown).

3.5. CDDP-induced mitochondrial dysfunction

Previously [15], we showed that CDDP causes mitochondrial dysfunction and onset of apoptosis in kidney cells. Therefore, we examined the effect of exposure of both cell lines to CDDP with regard to the mitochondrial function. The mitochondria of CC531s and -m2 cells were labelled with rhodamine-123. Subsequently, cells were exposed to CDDP and the mitochondrial function was analysed by flow cytometry at 0, 4, 8 and 12 h after exposure. At the start of the experiment (0 h) no difference

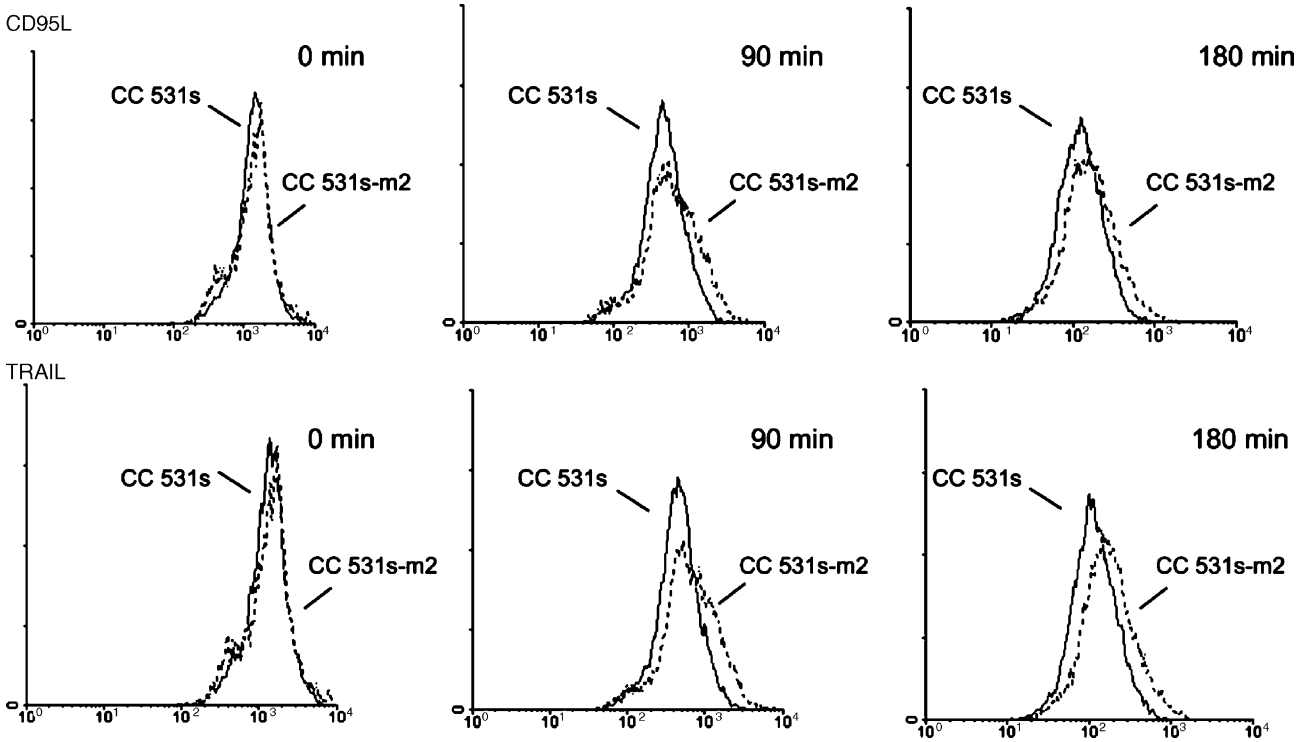

Fig. 5. Mitochondrial membrane potential decrease in C531s and -m2 cells upon CD95L- and TRAIL-signaling. CC531s and -m2 cells were seeded in 6-well plates and loaded with the fluorescent dye rhodamine-123. Next, the cells were co-incubated with CD95L- (A) and TRAIL-expressing effector cells (B) for the indicated time-periods. The target cells were harvested and analyzed by flow cytometry (cells in the life gate are shown). Data representative of three independent experiments are shown.

in rhodamine-123 intensity was found between the cell lines (Fig. 7). Also, after 4 h no difference was detected. In contrast, after 8 h of exposure, a greater loss of rhodamine-123 was seen in the CC531s cells as compared to the CC531s-m2 cells, indicating greater mitochondrial damage (median fluorescence intensity of 391 ± 56 in the CC531s cells versus 577 ± 85 in the CC531s-m2 cells). At 12 h the mitochondrial membrane potential had decreased slightly further in the CC531s cells, whereas in the CC531s-m2 cell line two subsets seem to be present based on the rhodamine-123 intensity; the subgroup with the lowest mitochondrial membrane potential however, still seemed to have a slightly higher potential than that of the CC531s cell line.

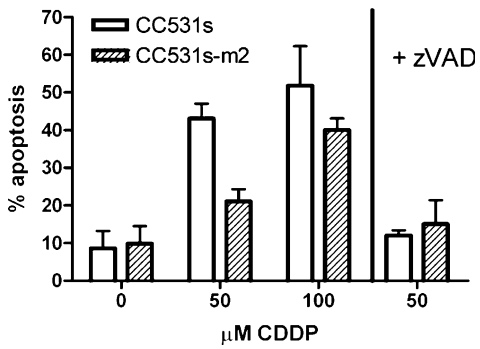

Fig. 6. Apoptosis-induction in CC531s and -m2 cells by CDDP. CC531s and -m2 cells were seeded in 6-well plates. Next, they were exposed to the indicated concentrations of CDDP in the presence or absence of zVAD-fmk for 17 h. Subsequently apoptosis was determined by flow cytometry. Statistically significant differences are marked with an asterisk. Mean values of three independent experiments are shown.

3.6. CDDP-induced caspase-activity

Clearly, the CC531s cell line was more sensitive to CDDP as compared to the CC531s-m2 cell line, using both mitochondrial damage as well as phosphatidyl-serine exposure as read-out. Since apoptosis is induced by the executioner caspase-3, we determined the activity of this caspase upon exposure of the cells to CDDP. Cells were exposed to CDDP for 17 h, harvested, lysed and the activity of caspase-3 was assessed by cleavage of the caspase-3 substrate Ac-DEVD-AMC. The results of this assay clearly confirmed the strongly decreased sensitivity of the CC531s-m2 cell line towards CDDP: exposure of CC531s-m2 cells to 50 or 100 μM CDDP caused a caspase-3 activity of 204 and 645 pmol AMC/min mg, respectively (Fig. 8). Exposure of CC531s cells to 50 μM CDDP caused 1450 pmol AMC/min mg caspase-activity, a seven-fold increase in activity as compared to the CC531s-m2 cell line. When exposed to 100 μM CDDP, the activity measured in the CC531s cells was 1298 pmol AMC/min mg, probably indicating that the kinetic of caspase-3 activity was passed its

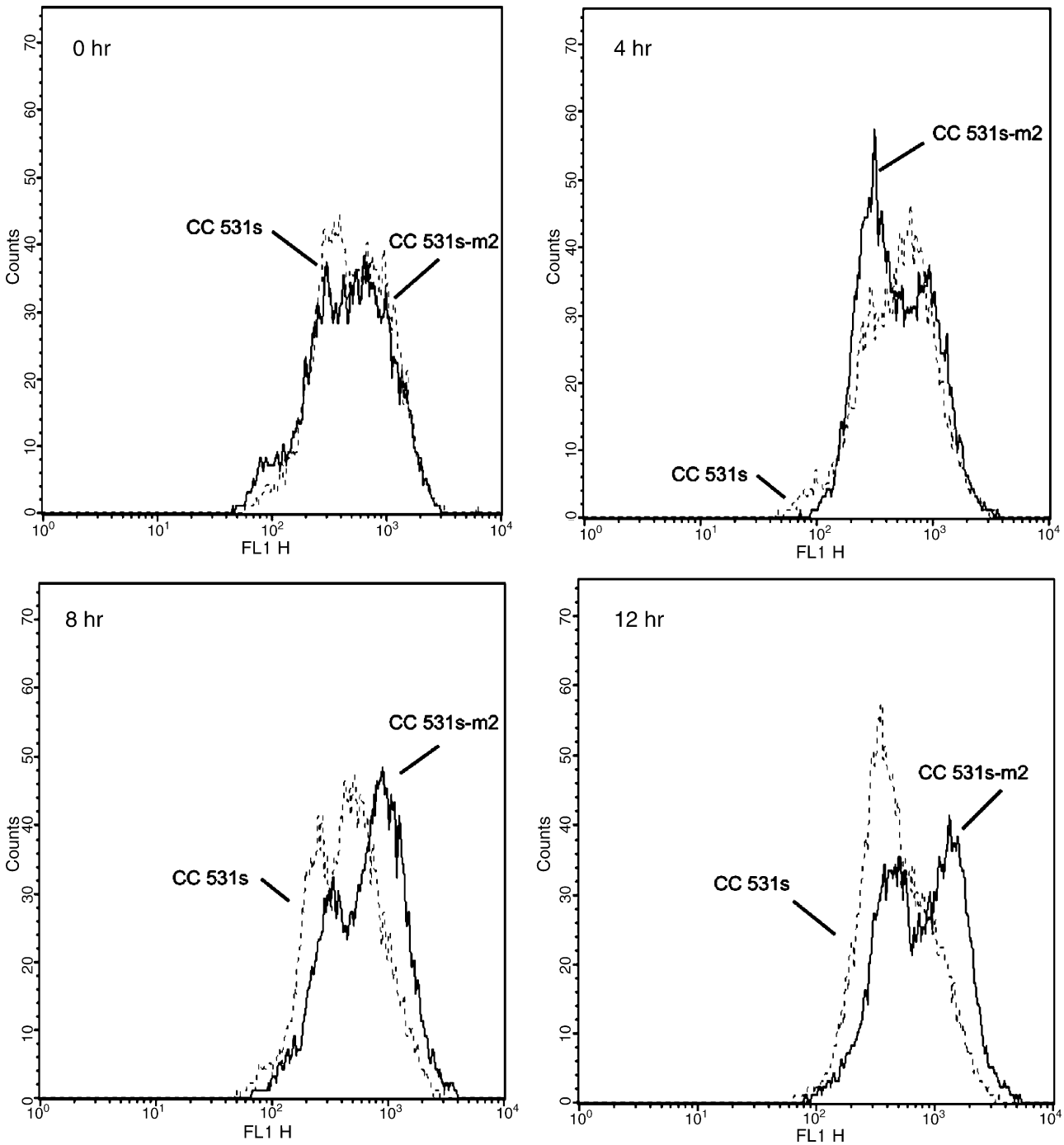

Fig. 7. Mitochondrial membrane potential decrease in C531s and -m2 cells upon CDDP-exposure. CC531s and -m2 cells were seeded in 6-well plates and loaded with the fluorescent dye rhodamine-123. Next, the cells were exposed to 50 μM CDDP for the indicated time-periods. Subsequently, the cells were harvested and analyzed by flow cytometry (cells in the life gate are shown). Data representative of three independent experiments are shown.

optimum. Nevertheless, the difference with the CC531s-m2 cell line was very large.

3.7. CDDP-induced caspase-cleavage

The activity of the executioner caspase-3 and the upstream caspase-8 were decreased in the CC531s-m2 cell line as compared to the parental CC531s cell line after CD95L- or TRAIL-exposure. After exposure to CDDP, the difference in caspase-3 activity was even much more profound. Finally, we checked whether the decreased caspase-3 activity was caused by less cleavage of caspase-3. To do so, we exposed CC531s and -m2 cells to 50 or 100 μM of CDDP for 17 h and subsequently western blotted the samples for active caspase-3 (the 10 and 20 kD fragments). As shown in Fig. 9, in the CC531s cells large quantities of active caspase-3 could be detected after exposure to both concentrations. In contrast, in CC531s-

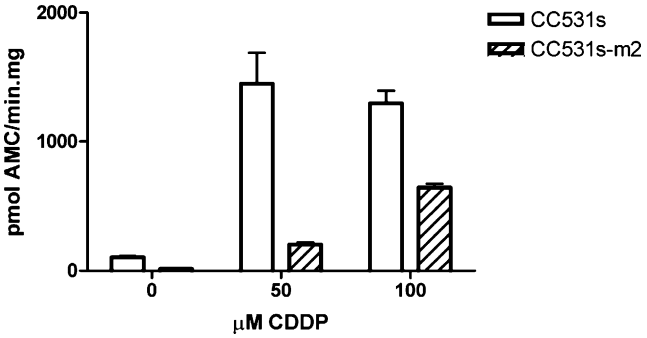

Fig. 8. Caspase-3 activation in CC531s and -m2 cells upon CDDP-exposure. CC531s and -m2 cells were seeded in 6 wells plates and exposed to the indicated concentrations of CDDP for 17 h. Next, the cells were harvested, lysed and the caspase-activity was determined by quantification of Ac-DEVD-AMC cleavage. Statistically significant differences are marked with an asterisk. Mean values of three independent experiments are shown.

m2 cells hardly any cleaved caspase-3 could be detected after exposure to 50 μM CDDP. When the concentration was increased to 100 μM more cleaved caspase-3 was detected, but not nearly as much as in the CC531s cells. (No difference was seen in the level of pro-caspase-3 in both cell types [16]).

4. Discussion

Previously [16] we described the CC531s-m2 cell line was capable of forming much more metastases in vivo then the parental cell line. Here, we further explored the pronounced decrease in CD95L- and TRAIL-mediated apoptosis observed in this cell line. The most straightforward explanation for a decreased response would be down-regulation of the membrane-expression of the death receptors or up-regulation of the decoy receptors. Therefore, we investigated the expression of the receptors, but we could find no differences (Fig. 1). It has been suggested that the subcellular localization of decoy receptors may be important in determining the effectiveness of TRAIL: TRAIL-insensitivity was found to be mediated by DcR1 when it was localized in the cytoplasm, in contrast to a nuclear localization of DcR1 in another cell line [23]. Thus, it cannot be excluded that a decreased response to TRAIL is due to differences in the subcellular localization of the (decoy-) receptors between the CC531s and CC531s-m2 cell line. However, this would not explain the insensitivity to CD95L. Therefore, it is more likely that there is a common cause for the decreased response to both TNF family members.

In a second set of experiments in which CC531s and CC531s-m2 cells were co-incubated with CD95L- and TRAIL-effector cells, we found significant differences between CC531s and -m2 cells. The level of caspase-8, -3 and -9 activities after CD95L- and TRAIL-exposure was much lower in CC531s-m2 cells than in CC531s cells. Also, a delayed decrease in mitochondrial membrane potential was observed. Although no differences in protein levels of the CD95L- and TRAIL-receptors were found using both western blotting [16] as well as flow cytometric approaches (Fig. 1), it can not be excluded that the in vivo selection of the CC531s-m2 cell line resulted in point mutations in the death receptors or their associated proteins, leading to reduced binding capacities. However, since the deceleration of the MMP was also seen upon exposure to CDDP, probably the prevention of mitochondrial damage caused the decreased caspase activities; less mitochondrial damage results in less active caspase-9, leading to less active caspase-3 which in its turn can cleave less death-substrates as well as less pro-caspase-8 which explains the differences seen in caspase-8 activity. This would require feedback activity of caspase-3 on caspase-8 as reported by Guo et al. [24]. An alternative explanation for the decreased susceptibility to CDDP-induced apoptosis might be an overexpression of P-glycoprotein (P-gp). However, DNA-chip analysis showed that both cell lines lack substantial RNA levels encoding P-glycoprotein (unpublished data).

Previously [16], we immunoblotted for bcl-2 levels in both cell types, but no differences were seen. However, relative big differences in protein expression are required to be detected with immunoblotting. It may be that a decrease in protein expression of 10% is sufficient to alter the susceptibility of cells to apoptosis, but not sufficient to be seen with western blotting. Another explanation may be the upregulation of other anti-apoptotic members of the bcl-2 family such as bcl-xl or downregulation of pro-apoptotic members of the bcl-2 family such as bax or bid. Recently, it was shown that Bax translocates to the mitochondrial membrane upon CDDP-induced apoptosis [13,25], causing reduction of the mitochondrial membrane potential [25]. However, in contrast to our findings, Kim et al. [25] reported that addition of the pan-caspase inhi-

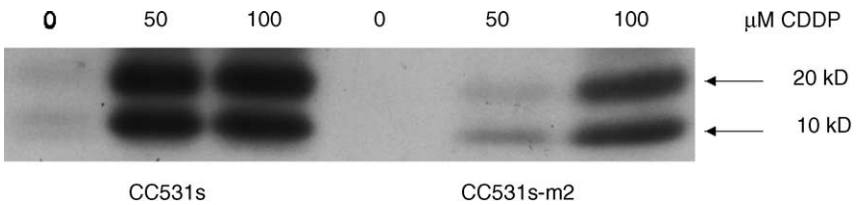

Fig. 9. Caspase-3 cleavage in CC531s and -m2 cells upon CDDP-exposure. CC531s and -m2 cells were seeded in 6-wells plates and exposed to the indicated concentrations of CDDP for 17 h. Next, the cells were harvested, lysed and an equal amount of protein was western blotted for cleaved caspase-3. Data representative of three independent experiments are shown.

bitor zVAD-fmk did not completely prevent apoptosis in their cells. This inconsistency may be explained by the cell types used: we used the rat colon carcinoma cell line CC531s, whereas Kim et al. [25] used the human hepatocellular carcinoma cell line Hep3B. One could speculate that in the Hep3B cell line a non-zVAD-fmk inhibitable protease can be activated that is not present in our CC531s cell line.

Our method to develop an immunosurveillance surviving cell type that rapidly metastasizes, resulted in a cell line with a decreased sensitivity towards apoptosis-inducing stimuli used by T- and NK-cells. Here, we showed that these cells were remarkably less susceptible to CDDP-induced apoptosis. Both death-inducing stimuli cause an altered response of the CC531s-m2 cell line as far upstream as the mitochondria and activation of caspase-8, suggesting that a common factor at the level of the mitochondria is responsible for the decreased sensitivity. This—as yet—unidentified factor may therefore be responsible for the survival of the cells during the process of metastasizing (i.e. surviving immunosurveillance) as well as the increased resistance of metastasizing cells towards anti-cancer drugs.

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