Research Article

Endonucleases induced TRAIL-insensitive apoptosis in ovarian carcinoma cells

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ARTICLE INFORMATION ABSTRACT

TRAIL induced apoptosis of tumor cells is currently entering phase II clinical settings, despite the fact that not all tumor types are sensitive to TRAIL. TRAIL resistance in ovarian carcinomas can be caused by a blockade upstream of the caspase 3 signaling cascade. We explored the ability of restriction endonucleases to directly digest DNA in vivo, thereby circumventing the caspase cascade. For this purpose, we delivered enzymatically active endonucleases via the cationic amphiphilic lipid SAINT-18\textsuperscript{b}:DOPE to both TRAIL-sensitive and insensitive ovarian carcinoma cells (OVCAR and SKOV-3, respectively). Functional nuclear localization after delivery of various endonucleases (BfiI, PvuII and NucA) was indicated by confocal microscopy and genomic cleavage analysis. For PvuII, analysis of mitochondrial damage demonstrated extensive apoptosis both in SKOV-3 and OVCAR. This study clearly demonstrates that cellular delivery of restriction endonucleases holds promise to serve as a novel therapeutic tool for the treatment of resistant ovarian carcinomas.

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Introduction

Dysfunction of apoptosis contributes to many diseases including cancer. Two major apoptotic pathways have been identified. The receptor mediated pathway, or death-receptor pathway, is triggered by the death-receptor super family and responds mainly to extracellular stimuli. The receptor independent, or mitochondrial pathway, is activated by intracellular signals such as DNA damage and growth factor depletion. These two pathways converge at the level of caspase 3 activation, resulting in the execution of apoptosis [1,2]. Anticancer therapies currently in clinical use induce apoptosis of the target cell either way. A potent death-receptor mediating anticancer agent is TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), which has been shown to induce apoptosis in many tumor cells via binding to death-receptors 4 and 5. Because TRAIL exhibits no apoptotic activity towards normal
human cells [3–7], it is an attractive candidate for cancer treatment. However, some tumor types, including some ovarian carcinomas, are resistant to TRAIL [8–18].

Ovarian cancer represents the most fatal gynecologic type of cancer in the world. Current treatments involve surgery followed by chemotherapy. However, development of chemo-resistance is frequently observed posing a serious limitation to the success of current therapeutic approaches. Therefore, development of novel therapeutic entities, such as TRAIL, is highly warranted. However, resistance of ovarian carcinomas to TRAIL has been reported, and is partly associated with defects located upstream of the caspase 3 signaling cascade [10]. Treatment strategies for TRAIL-resistant ovarian carcinomas should therefore include a strategy to bypass this blockade. Caspase 3 activation is followed by DNA cleavage effected by endogenous nucleases leading to DNA laddering, a feature of apoptotic cells. Direct induction of DNA cleavage therefore provides a good alternative to circumvent the caspase 3 activation step. In this context, endogenous endonucleases, although extensively used as laboratory tools for molecular biological purposes, have not been used as pharmaceutical agents.

So far, no direct delivery of restriction endonucleases has been investigated as anticancer agent. In this study, we demonstrate functional delivery of restriction endonucleases to ovarian carcinoma cell lines using SAINT-18®:DOPE. We tested the randomly cutting endonuclease NucA, and its inactive forms [19], and two six-base-pair cutting restriction endonucleases PvuII and BfiI including the mutant variant BfiI-K107A [20,21,21] and identified especially PvuII as a strong inducer of apoptosis in both TRAIL-sensitive and insensitive cell lines.

Materials and methods

Reagents

PvuII, which cleaves the palindromic sequence 5′-CAGCTG-3′ in a Mg2+-dependent manner resulting in double strand breaks with blunt-ends [21] and NucA, a non-specific endonuclease from the ββα-blunt-ends endonuclease super family, which nicks the DNA [19], its variant NucA-H124A, and the inactive nuclease-inhibitor complex NucA–NuiA, were kindly provided by Dr. W. Wende (Gießen, Germany). The mutant NucA-H124A possesses a His to Ala mutation at residue 124, positioned in the catalytic centre of the enzyme [19]. In the inactive nuclease/inhibitor complex NucA–NuiA, the inhibitor NuiA binds NucA with picomolar affinity, thereby preventing digestion of nucleic acids by NucA. BfiI which cleaves the DNA at fixed positions downstream of an asymmetric sequence (5′-ACTGGG-3′) in a double strand break, but acts without requiring metal ions [20] and its mutant variant BfiI-K107A, which possesses a Lys to Ala mutation at residue 107, were kindly provided by V. Siksnys (Vilnius, Lithuania).

SAINT-18®:DOPE (commercially available as SAINT:MIX) was purchased from Synvolux Therapeutics Inc. (Groningen, The Netherlands). The caspase inhibitor Z-VAD-fmk was obtained from Calbiochem (San Diego, CA, USA). Actinomycin D was obtained from USB Corporation (Cleveland, USA). DioC6 (3,3′-dihexylocarbocyanine) was purchased from Molecular Probes (Eugene, OR, USA). scFv425:sTRAIL was kindly provided by Dr. W. Helfrich (University Medical Center Groningen, the Netherlands).

Cell culture

The human ovarian cancer cell lines SKOV-3 and OVCAR were obtained from the ATCC (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 50 μg/ml gentamycine sulfate, 2 mM l-glutamine, 10% FBS (BioWhittaker Inc, Walkersville, MD) at 37 °C under humidified conditions. Primary isolated Fetal Lung Fibroblasts (FLF) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 50 μg/ml gentamycine sulfate, 2 mM l-glutamine, 10% FBS (BioWhittaker Inc, Walkersville, MD) at 37 °C under humidified conditions. HUVEC (human umbilical vein endothelial cells) were obtained from the UMCG Endothelial Cell Facility and cultured in RPMI 1640 supplemented with 20% heat inactivated FBS, 2 mM l-glutamine, 5 U/ml heparin (Leo, Weesp, The Netherlands), 100 U/ml penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100 μg/ml streptomycin, and 50 μg/ml gentamycin.

Fig. 1 – Cleavage pattern of Restriction endonucleases on genomic DNA after delivery of restriction enzymes using SAINT-18®:DOPE. SKOV-3 cells were proffected for indicated time points with: (a) 10 μg of the non-specific endonuclease NucA, and inactive forms of the enzyme for 48 h. lane 1, Blanko; 2, SD; 3, SD+NucA; 4, SD+H124A; 5, SD+NucD; 6, Actinomycin D. (b) 2 μg BfiI (type IIS REase) and the BfiI variant K107A for 4 h. 1, Marker; 2, Blanko; 3, SD; 4, BfiI; 5, SD+BfiI; 6, K107A; 7, SD+K107A. (c) 2 μg BfiI (type IIS REase) and the BfiI variant K107A for 24 h. lane 1, Blanko; 2, SD; 3, BfiI; 4, SD+BfiI; 5, K107A; 6, SD+K107A. (d) 1 μg of PvuII (type IIP REase), BfiI (type IIS REase) and NucA for 48 h. lane 1, Blanko; 2, SD; 3, PvuII; 4, SD+PvuII; 5, BfiI; 6, SD+BfiI; 7, NucA; 8, SD+NucA; 9, Act.D; 10, scFv425:sTRAIL. After proffection, the fragmented DNA was sheared and analyzed by 1.3% agarose gel electrophoresis as described in “Material and methods”.

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crude endothelial cell growth factor (home isolated) in 1% gelatin-coated tissue culture flasks at 37 °C under humidified conditions.

Anonymized primary human skin fibroblasts were kindly provided by Dr. K.E. Niezen-Koning (University Medical Center Groningen, the Netherlands) and were cultured in F10HAM medium supplemented with 10% FCS and 2% Penicillin–Streptomycin (Invitrogen, Breda, The Netherlands).

Cleavage of endogenous genomic DNA

SAINT-18®:DOPE (SD) was used to deliver restriction endonucleases as protein (profection, in detailed described in [22]). Cells were seeded at 0.3×10⁶/6-well and protein delivery was performed at 50–80% confluence. Cells were treated with 1, 2 or 5 μg endonuclease alone or in complex with SD. As positive controls for inducing cell death Actinomycin D at a final concentration of 0.2 μg/ml in serum-containing medium and scFv425:sTRAIL [23] at a final concentration 2.7 μg/ml were used. The cells were incubated for 24 or 48 h at 37 °C in humidified 5% CO₂. To inhibit caspase activity, 20 μM (final concentration) Z-VAD-fmk was added.

Apoptosis analysis

DNA fragmentation analysis

Cells were collected 48 h after treatment and resuspended in lysis buffer (0.5% N-Lauroyl sarcosine (Sigma), 0.5 mg/ml RNase A, 1 mg/ml Proteinase K (Invitrogen) in 50 mM Tris–HCl, pH, 8.0) and incubated at 50 °C for 2 h. The samples were sheared, loaded on a 1.3% agarose gel and run for 15 min at 100 V (dry, after 15 min 1× TAE was added). DNA was visualized by ethidium bromide fluorescence on exposure to ultraviolet light.

For analysis of DNA fragmentation by flow cytometry, cells were collected and pellets resuspended in 100 μl PBS. After addition of 3 ml ice-cold (−20 °C) 70% EtOH, cells were incubated for 1 h at 4 °C, washed twice with PBS and centrifuged for 5 min at 1800 rpm. After which 100 μl 1:10 RNase buffer (10 mM Tris, 15 mM NaCl, RNase

Fig. 2 – Apoptosis induction after delivery of endonucleases with SAINT-18®:DOPE as determined by loss of mitochondrial potential. OVCAR (a) and SKOV-3 (b) were profected with the endonucleases NucA, Pvull and BfiI (5 μg) in complex with SD. After 48 h, the cells were stained with DioC6 and analyzed by flow cytometry.

Fig. 3 – Intranuclear delivery as assessed by confocal imaging. SKOV-3 cells were profected with 0.1 μg PvuII-Alexa 488 with or without SD. 48 h after treatment, cells were fixed and nuclei were counterstained using the dye ToPro-3. Cells were imaged using a Leica TCS SP2 (AOBS) inverted confocal microscope.
(10 mg/ml) was added followed by 10 min of boiling (to deactivate DNases). After 30 min of incubation at 37 °C, 200 μl solution (0.1 μg/μl in PBS) was added. After another 30 min incubation at 4 °C, cells were analyzed using a Calibur flow cytometer.

**Mitochondrial-membrane potential analysis**

Apoptosis was assessed by loss of mitochondrial-membrane potential using the cell permanent green-fluorescent lipophilic dye DioC6. Cells were harvested by centrifugation (1500 rpm for 5 min), incubated for 20 min at 37 °C with 0.1 mM DioC6 in fresh medium, harvested (1500 rpm for 5 min), resuspended in 250 μl fresh medium and analyzed for DioC6 staining using a Calibur flow cytometer (Becton Dickinson Biosciences, Breda, The Netherlands).

**Confocal microscopy**

To visualize intracellular delivery of the restriction endonuclease PvuII confocal microscopy was performed. For profection, SKOV-3 cells were seeded 0.3×10^5/8-well (8 well 1 μ-Slide IBIDI treat from Ibidi) and protein delivery was performed at 40% confluency. Cells were treated with 0.1 μg PvuII labelled with the fluorescent dye Alexa-488 with or without SD. 48 h after profection cells were fixed in 4% formaldehyde/DMEM+ for 15 min at 37 °C, after washing cells were permeabilized with 0.2% Triton X-100/PBS for 10 min at RT. Finally, nuclei were counterstained with To-Pro3 (Invitrogen)/PBS solution (1:5000) for 15 min at RT. Cells were imaged using a Leica TCS SP2 (AOBS) inverted confocal microscope with a 40× optical lens. Analysis was performed using Image J software.

**Phosphorylated histone 2AX (γH2AX) detection**

**γH2AX Western Blot**

Cells were lysed in 200 μl buffer (20 mM Tris–HCl, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 0.05% SDS, 0.5% NP-40, 1 mM Phenylmethylsulfonyl Fluoride (PMSF), 10 μg/ml Aprotinin, 10 μg/ml Leupeptin and loading buffer with 2-mercapto-ethanol). Proteins were separated by SDS-PAGE and electroblotted onto 0.45 μm nitrocellulose transfer membranes (Schleicher and Schuell, Dassel, Germany). Immunodetection was done with phosphorylated Histone 2AX (Phospho-Histone H2AX (Ser139) (20E3) Rabbit mAb (Alexa Fluor 488 conjugate) #9719, Cell Signaling, Leiden, The Netherlands), or 1:1000 of caspase-8 antibody (1C12) (Cell Signaling, #9746) followed by RoM-PO and visualized using the ECL chemoluminescence detection kit (Pierce, Rockford, IL).

**γH2AX FACS analysis**

At indicated time points, cells were harvested and washed with PBS. Cells were fixed in 4% Formaldehyde (Merck)/PBS for 10 min at 37 °C. After addition of 1 ml PBS and chilling on ice for 1 min, cells were washed and permeabilized in 90% methanol/PBS for 30 min on ice. Staining of phosphorylated H2AX was performed as follows: permeabilized cells were washed by addition of 2 ml of incubation buffer (0.5% BSA in PBS). Cells were resuspended in 85 μl incubation buffer followed by addition of 10 μl of conjugated antibody, 4 μl of PI (final conc. 10 μg/ml; Nexins, Kattendijke, The Netherlands) and 1 μl of RNase (final conc. 100 μg/ml) and were incubated for 30 min in the dark at RT. Cells were washed again with incubation buffer, resuspended in 200 μl PBS and analyzed for

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**Fig. 4** – In vitro DNA fragmentation after profection of PvuII using SAINT-18®:DOPE. SKOV-3 cells were profected with 10 μg of PvuII for 48 h. After profection, FACS analysis was performed on SKOV-3 cells after treatment with SD+PvuII (10 μg) to confirm intranuclear DNA fragmentation after 48 h. The peak arising in front of the G0 peak (SD + 10 μg) represents DNA fragments.

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phosphorylated H2AX staining using a Calibur flow cytometer. Graphs were made using WinMDI software.

Statistical analysis

Statistical comparisons between untreated cells (BL) and cells treated with SD + restriction endonuclease, Actinomycin D or scFv425-TRAIL were performed using the paired t-test. Statistical significance was indicated by $P < 0.05$.

Results

Analysis of genomic DNA cleavage by protected restriction endonucleases

Functional delivery of different types of restriction endonucleases was studied using SAINT-18®:DOPE as delivery device. We tested the randomly cutting non-specific endonuclease NucA, and its less active forms, as well as three less frequently cutting type II REases, PvuII and BfiI and the BfiI variant K107A. The restriction endonucleases were delivered into SKOV-3 cells with or without SD and DNA fragmentation gel analysis was performed on DNA isolated from treated cells (Fig. 1). 48 h after profection, NucA was able to completely fragment the DNA (Fig. 1a). BfiI caused frag-
mentation of the DNA already 4 h after profection, but the nicks seemed to be repaired after 24 h (Figs. 1b and c respectively).

PvuII was most effective in inducing DNA fragmentation 48 h after profection (Fig. 1d). These data indicate that the endonu-
clases are able to exert their catalytic function after intracellular delivery.

Apo
totic effects of endonucleases after delivery with SD

After establishing intracellular and functional delivery of the restriction endonucleases, we compared the observed restriction endonuclease-mediated apoptosis with apoptosis induced by Actinomycin D and scFv425:sTRAIL [23] (Fig. 2). In concordance with literature, the ovarian carcinoma cell lines used in this study respond differently to the potent apoptosis inducer TRAIL: SKOV-3 is TRAIL-resistant, whereas OVCAR is sensitive for TRAIL induced apoptosis. scFv425:sTRAIL was able to induce apoptosis in the TRAIL-sensitive ovarian carcinoma cell line OVCAR (73% ± 0.7%), but not in SKOV-3 cells (12% ± 4.2%). The positive control Act.D led to induction of apoptosis in both cell lines (48% ± 3.8% in OVCAR and 35% ± 18% in SKOV-3).

Treatment of OVCAR (Fig. 2a) and SKOV-3 (Fig. 2b) cells with NucA:SD hardly induced any apoptosis, whereas treatment with BfiI:SD induced apoptosis in 54 (± 5.3%) and 21% (± 4%) of OVCAR and SKOV-3 cells, respectively. Treatment of PvuII:SD induced the highest rates of apoptosis in both OVCAR (50% ± 0.5%) and SKOV-3 cells (77% ± 3.4%). These findings expose PvuII, as low as 1 μg/ml (data not shown), as an efficient endonuclease for the induction of apoptosis also in the TRAIL-resistant cell line.

Fig. 5 – Flow cytometric analysis of phosphorylated H2AX in SKOV-3 treated with PvuII with or without SAINT-18®:DOPE. SKOV-3 cells were treated with PvuII with or without SD. At indicated time points, cells were collected and stained for phosphorylated H2AX and PI to counterstain cellular DNA. FACS analysis was performed to analyse phosphorylation of H2AX. Graphs were made using WinMDI software. Panel a shows the results 4, 8, 24 and 30 after treatment with PvuII:SD. Panel b shows the results 30 h after treatment with PvuII, PvuII:SD and Act.D. Cut off for true γH2AX positive cells is shown by line at 10^2 MFI.
Analysis of nuclear localization of the restriction endonuclease PvuII

To demonstrate that the observed cell death is due to nuclear activity of the restriction endonucleases, we visualized the subcellular localization of PvuII labelled with the fluorescent dye Alexa 488 using confocal microscopy. The pictures indicate nuclear localization of the restriction endonuclease PvuII-Alexa 488 using ToPro-3 to counterstain the nucleus (Fig. 3). PvuII-Alexa 488 alone was also efficiently taken up by cells, but remained localized in the cytoplasm. The presence of vesicles were observed by both PvuII-Alexa 488 + SD and PvuII-Alexa 488 alone, suggesting the involvement of an endocytic pathway in the internalization.

To further confirm functional intranuclear delivery, PvuII was delivered into SKOV-3 cells using SD and cells were analyzed by intranuclear DNA fragmentation. FACS analysis of cellular DNA content, demonstrated fragmentation 48 h after delivery of PvuII in SKOV-3. Treatment of the cells with SD or PvuII alone did not cause fragmentation of the DNA (Fig. 4).

Next, we investigated the kinetics of PvuII delivery in SKOV-3. Upon induction of double strand breaks, the histone H2AX becomes rapidly phosphorylated and is recruited to the site of DNA damage. SKOV-3 cells were analyzed for phosphorylated H2AX using FACS analysis, 4, 8, 24 and 30 h after profection with PvuII (Fig. 5). As untreated cells are known to contain background levels of γH2AX, we set the cut off for γH2AX at 10^{-5} (0.1% of normal cells). Delivery of PvuII resulted in phosphorylation of H2AX already 4 h after profection: 32% of the cells were positive for phosphorylated H2AX and this percentage increased to 50% for 30 h after treatment (Fig. 5a). Actinomycin D treatment also resulted in phosphorylated H2AX and increased over time although in a lesser extent (to 9% after 4 h (data not shown) and 34% after 30 h) (Fig. 5b).

Using Western Blot analysis (Fig. 6) we showed that scFv425: sTRAIL treatment after 48 h resulted in phosphorylation of H2AX and in caspase 8 activation in the TRAIL-sensitive cell line OVCAR, but not in the TRAIL-resistant cell line SKOV-3. Treatment with PvuII and Actinomycin D led to phosphorylation of H2AX and to

![Fig. 6](image-url)
caspase 8 activation in both cell lines. These data demonstrate that we efficiently induced double strand breaks and caspase activation for PvuII as well as for Actinomycin D in both SKOV-3 and OVCAR, showing that PvuII induces apoptosis in a TRAIL independent manner.

To further demonstrate this, we performed PvuII profection experiments in SKOV-3 and OVCAR cells with or without the total caspase inhibitor Z-VAD-fmk. After 48 h, cells were harvested and analyzed for induction of apoptosis by FACS analysis (Fig. 7). Treatment with PvuII:SD resulted in induction of apoptosis in both OVCAR as well as SKOV-3 cells even in the presence of Z-VAD-fmk. Treatment with scFv425-sTRAIL induced apoptosis and was efficiently blocked in the TRAIL-sensitive cell line OVCAR in the presence of the caspase inhibitor.

Effect of PvuII on normal cells

PvuII successfully induces apoptosis in both TRAIL-sensitive and TRAIL-resistant ovarian carcinomas, however for a clinical setting the potential toxic effect towards normal cells needs to be assessed. Fig. 8 depicts the results 48 h after profection of 1 μg PvuII in primary human skin fibroblasts (Fig. 8a), HUVECs (Fig. 8b) and Fetal Lung Fibroblasts (Fig. 8c). After treatment with PvuII:SD or scFv425-sTRAIL, no apoptosis induction was observed after 48 h (7%) in primary human skin fibroblasts, although confocal microscopy confirmed nuclear localization. Actinomycin D led to
an induction of apoptosis of 57%. In HUVEC cells, already a high background level of apoptosis was observed (35%). Treatment with PvuII:SD only slightly increased apoptosis (50%), while again no effect was observed for scFv425:sTRAIL. Actinomycin D did induce apoptosis in 90% of the HUVECs. Fetal Lung Fibroblast cells showed 60% apoptosis after treatment with PvuII:SD. Actinomycin D led to a strong induction of apoptosis (75%). Interestingly, also scFv425:sTRAIL showed apoptotic activity towards Fetal Lung Fibroblast cells (30%). These data demonstrate that treatment with PvuII:SD does induce apoptosis in HUVEC and Fetal Lung Fibroblast cells, but not in primary human skin fibroblasts. While for the carcinoma cells up to 2 fold higher apoptosis was induced by PvuII compared to Actinomycin D, the extent of apoptosis in “normal” cells, however, is lower than the apoptosis induced by Actinomycin D.

Discussion

Cells and tissues in the human body are maintained in homeostasis by the physiological process of apoptosis. Malfunction of apoptosis is considered to be an important step in the development of cancer and metastasis. Various anticancer therapies thus aim to induce apoptosis of the tumor cells. Although TRAIL is a potent and specific apoptosis inducer and as such a promising anticancer approach for otherwise resistant tumors.

The data presented here clearly demonstrate efficient delivery of the restriction endonucleases using SD, resulting in apoptosis. The apoptotic activity, however, was also observed to some extent for normal cells, requiring targeted delivery for in vivo application. Coupling of antibodies or peptides to SD (manuscript in preparation) and other liposomal formulations has shown promise for targeted delivery strategies towards cancer cells or to sites of chronic inflammation [31–33]. As such targeting strategies are being optimized, direct delivery of restriction endonucleases offer a promising approach of inducing targeted cell death for otherwise resistant tumors.

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