Apoptin gene therapy in head and neck cancer

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Apoptin enhances radiation induced cell death in poorly responding head and neck squamous cell carcinoma cells

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Abstract

Treatment of head and neck cancers is still rather poor and worldwide new treatment options are sought. Sensitizing radioresistant tumors by combining irradiation with other therapeutics to induce apoptosis are widely investigated. We examined whether chicken anemia virus derived apoptin protein would have a beneficial effect on irradiation of radiosensitive SCC61 and radioresistant SQD9 human head and neck squamous carcinoma cell lines. In both cell lines, concurrent exposure to irradiation and apoptin resulted in analyzed mitochondrial cytochrome c release and in cleavage of caspase-3, whereas irradiation alone of SQD9 cells under identical conditions did not. Moreover, in comparison to the irradiation only treatment the synchronized treatment of apoptin and irradiation resulted in increased cell death in especially the radioresistant SQD9 cells, as measured by means of a colony survival assay. Our data reveal that apoptin treatment represents an effective way for enhancing radiotherapy of tumors responding poorly to radiotherapy.

Introduction

The worldwide incidence of head and neck squamous cell carcinoma (HNSCC) is estimated at 500.000 cases each year[1]. Despite advances in diagnosis and treatment the 5-year survival rate for e.g. larynx cancer only progressed from 53% in the mid seventies to 56% in the mid nineties[2]. For several sub sites in the head and neck treatment options are surgery, radiotherapy and chemotherapy and combinations thereof. The prognosis of nasopharynx-, oropharynx- and hypopharynx cancer has improved during the last decades in part due to combinations of chemotherapy and radiotherapy[3].

In spite of these advances the prognosis of HNSCC remains poor, in part due to the resistance of cancer cells to radiotherapy and chemotherapy[4]. Lately, gene therapy has been successful in treatment of head and neck and other cancers via induction of apoptosis[5-7]. The apoptotic pathways are controlled by several pro-apoptotic and anti-apoptotic proteins, which might be defective or over-stimulated in cancer cells, respectively. Such imbalanced apoptosis machinery might cause resistance to radio-/chemotherapy, but remains still sensitive to specific gene products. Several of these gene therapeutic agents are even known to sensitize chemotherapy and radiotherapy[8,9].

Apoptin, a chicken anemia virus derived protein has shown to effectively kill tumor cells both *in-vitro* and *in-vivo*[10]. Cell death by apoptin is p53 independent[11] and normal cells are not affected by apoptin[12]. The level of impact in the apoptotic pathway seems to be different in comparison to radio- and chemotherapy[13].

Here, we report our studies on the anti-tumor effect of a combination of radio-and apoptin gene therapy in radiosensitive and radioresistant squamous head and neck carcinoma cells. The combinatorial apoptin-irradiation treatment has a beneficial effect on the cell kill of radioresistant squamous head and neck carcinoma cells.

Materials and Methods

Cell culture.

The radiosensitive SCC61 and the more radioresistant SQD9 human head and neck squamous carcinoma cell lines known upon irradiation to undergo significantly less cell death[14] are a kind gift from A.C. Begg, Netherlands Cancer Institute, Amsterdam, the Netherlands. The cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin and streptomycin (Life Technologies, Rockville, Md.). Cultures were incubated at 37ºC and passed by trypsinization. The cells were used for the various experiments when they were in the exponential growth phase.

Viruses and virus techniques.

The recombinant adenoviral vectors expressing apoptin (AdMLP. apoptin) or encoding β-galactosidase (AdCMV.LacZ) revealing similar transduction and transgenic expression levels were generated as described previously[15]. The recombinant viruses were purified by double CsCl-density centrifugation. Titers of the viral stocks were determined by plaque assay on 911 cells. Virus was aliquoted and stored in sucrose buffer (140 mM NaCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 20 mM $MgCl_2$, and 5% sucrose) at -80°C. The cells were infected with the recombinant adenoviral vectors at a MOI of 5, which revealed under the used conditions a near complete transduction efficiency[15].

Irradiation.

Exponentially growing cells were seeded in 10-cm dishes or 6-well plates. Twenty-four hours later, the cells were exposed to recombinant viruses AdMLP.apoptin or AdCMV.LacZ at an MOI of 5 at 37°C. Fresh medium (2 mL) was then added to each well and incubation was continued for 24 hrs. One day after the viral infection, cells were irradiated using a 6 MV linear

accelerator. Doses were given in a single fraction of 2–6 Gy. After irradiation, the dishes were immediately returned to the incubator.

Cytochrome c assay.

First treated cells were subcellularly fractionated. The cell monolayer (10^6 cells/10-cm dish) was rinsed twice with ice-cold PBS, scraped and centrifuged at 2000 g for 5 min. The cells were resuspended in cell extraction buffer (300 mM sucrose, 10 mM Hepes [pH 7.4], 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 100 µM cytochalasin B), left on ice for 30 min, and homogenized by means of vortexing the lysates. Unbroken cells and nuclei were pelleted by centrifugation for 5 min at 2000 *g*. Heavy membranes were removed from the resulting supernatant by centrifugation for 5 min at 14000 *g*. The resulting supernatant is the crude cytosolic fraction. All samples were frozen in liquid nitrogen and stored at -80° C until analysis by sodiumdodecylsulfide-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the cytoplasmic cytochrome *c* levels were determined. Equal amounts of cytosolic protein extract were boiled in reducing sample buffer for 5 min, loaded in a lane of a sodiumdodecylsulfide-15% polyacrylamide (SDS-PAA) gel, and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hour in PBST-buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat dried milk. Subsequently, the blots were then incubated during overnight with monoclonal antibody 7H8.2C12 (Pharmingen; dilution 1:1000) to detect cytochrome c. Following overnight incubation, membranes were washed and incubated for 1 h at room temperature with secondary antibody. Membranes were washed three more times and incubated for 5 min at room temperature with Super Signal HRP substrate (Pierce). Positive signals were visualized by enhanced chemiluminescence according to the manufacturer's protocol (Amersham, Roosendaal, the Netherlands).

Caspase-3 analysis.

Cells (8×10^5/dish) were grown in 60-mm plates and treated as follows: Cells were collected by gentle scraping, washed once with ice-cold PBS, and lysed in lysis-buffer (PBS, 1% Triton X-100, 10% glycerol) containing protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Insoluble material was removed by centrifugation at 2,000 g for 15 min at 4°C. Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions. Fifty micrograms of protein per sample was fractionated by NuPAGE 4–12% Bis–Tris gels (InVitrogen, Carlsbad, CA, USA) and transferred onto nitrocellulose membranes (Bio-Rad, Veenendaal the Netherlands). Following transfer, membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk and incubated overnight at 4°C with monoclonal anti-active caspase-3 antibody C92-605 (dilution 1:5000; Pharmingen, San Diego, CA, USA). Further treatment and analysis of the membranes is identical as described for the cytochrome c assay.

Clonogenic survival.

The effectiveness of the combination of apoptin and ionizing radiation was assessed by clonogenic assays. Subconfluent cell cultures were treated with either AdMLP.apoptin or AdCMV.LacZ or mock-infected. Twenty-four hours later the medium was refreshed and the cells were non-exposed or exposed to different doses of ionizing irradiation, and incubated for another 24 hours at 37°C. Next, the cells were trypsinized and counted. Known numbers were then replated in 10-cm dishes and returned to the incubator to allow macroscopic colony development for 12 days. The individual colonies were fixed and stained with a solution containing 0.25% crystal violet and 10% ethanol for 10 minutes. Colonies containing more than 50 cells were scored and the percentages of surviving cells were calculated. The relative surviving fraction after each treatment was calculated based on the survival

of the fraction of non-irradiated control treated cells, which was set arbitrarily 100%.


Results

Apoptin induces apoptosis in both radiosensitive and radioresistant HNSCC cells.

To examine whether apoptin sensitizes radiotherapy in HNSCC cells, we have analyzed its effect on irradiation of the radiosensitive SCC61 cells and of the radioresistant SQD9 cells. First, we have analyzed the level of apoptin-induced cell death in these two HNSCC cells to examine whether also apoptin-induced cell death is hampered in radioresistant HNSCC cells in comparison to radiosensitive SCC61 cells.

Both SCC61 and SQD9 cell lines were treated with AdMLP.apoptin encoding apoptin or with the negative control AdCMV.LacZ encoding β-galactosidase. Four days after infection, the cells were fixed and the expression of apoptin and β-galactosidase was analyzed by indirect immunofluorescence using specific antibodies. The DNA of the cells was stained with DAPI, which stains intact DNA regularly but DNA of dead cells irregularly[16]. Apoptin-positive radiosensitive SCC61 cells contained for 76% a cell death DNA morphology; whereas that holds true for 71% of the apoptin-positive radioresistant SQD9 cells (Figure 1). Over-expression of β-galactosidase led to a slightly higher cell death level of 14% and 13% for SCC61 and SQD9 cells in comparison to non-expressing cells (7% and 8%, respectively). Therefore, we conclude that both the radiosensitive SCC61 cells and the radioresistant SQD9 cells are to a similar level sensitive to apoptin-induced cell death. One might assume that the blockage preventing radiation-induced cell death in radioresistant SQD9 cells seems to be circumvented by apoptin.

Figure 1
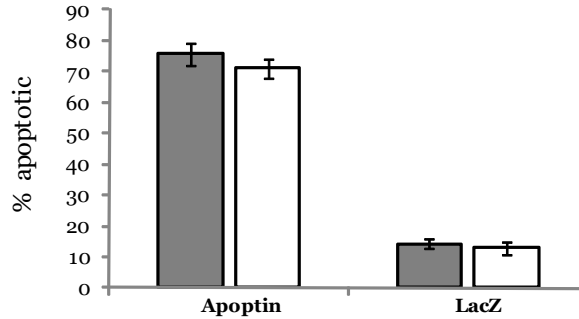
Apoptin induces cell death in radiosensitive SCC61 (gray bars) and radio-resistant SQD9 (open bars) to a similar level. The cells were infected with AdMLP.apoptin (left panel) encoding apoptin or with the negative control AdCMV.LacZ (right panel) encoding the non-apoptotic β-galactosidase. Four days after infection, the cells were fixed and analyzed by indirect immunofluorescence. The percentage of apoptin- or β-galactosidase-positive cells that stained abnormally with DAPI is given as a relative measure for cell death induction. Two independent experiments were carried out. Per experiment, at least 100 cells that were apoptin- or β-galactosidase-positive were examined.

*Combinatorial effect of apoptin and radiation on the survival rate of radioresistant SQD9 cells.*

Next, we determined whether apoptin can increase the sensitivity of tumor cells to radiation resulting in a decrease of clonogenic cell survival. SCC61 and SQD9 cells were exposed to AdMLP.apoptin or the negative control AdCMV.LacZ during 1 day and then treated with 0, 2, 4 or 6 Gy of radiation. After another day, the cells were plated and incubated for 12 days to determine the colony formation as described in the Materials and Methods section.

The dose-response curves for SQD9 and SCC61 cells in response to irradiation with apoptin or β-galactosidase treatment are presented in Figure 2. In comparison with the radiosensitive SCC61 cells, radiation induced a moderate response in the radioresistant SQD9 cells. For instance, 6 Gy radiation and β-galactosidase treatment of SCC61 cells resulted in a colony survival rate of approximately 10% (Figure 2A), whereas for SQD9 cells a colony survival rate of only nearly 30% was observed (Figure 2B).

At the different radiation dosages, the radiosensitive SCC61 cells

revealed a slightly enhancing effect of combined radiation and apoptin treatment in comparison to irradiation combined with the negative β-galactosidase control (Figure 2A). In contrast, SQD9 cells showed a clear additional effect of apoptin combined with radiation. For instance, SQD9 cells treated with apoptin and 6 Gy radiation revealed a colony survival rate of approximately 10%, whereas SQD9 cells treated with 6 Gy radiation and the negative β-galactosidase control showed a rate of nearly 30% (Figure 2B). These results suggest that apoptin sensitizes the radioresistant SQD9 cells to radiation or that combined radiation and apoptin treatment has an additive cytototoxic effect on the radioresistant HNSCC cells such as SQD9 cells.
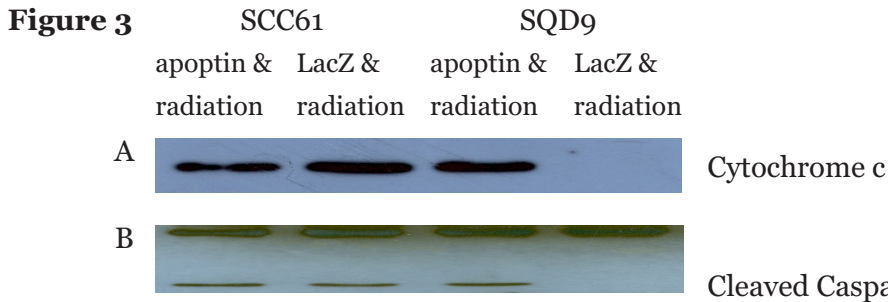
Figure 2
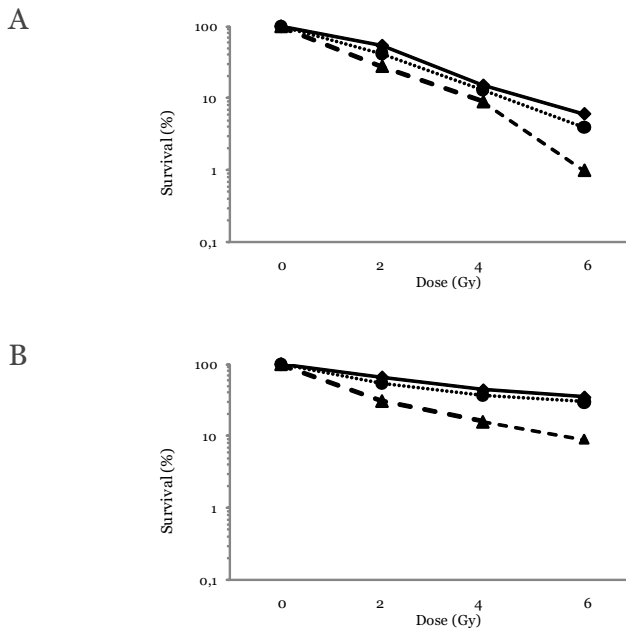
Apoptin enhances irradiation-induced cell death in radioresistant HNSCC cells. Human radiosensitive SSC61 (panel A) and radioresistant SQD9 (panel B) cells were either irradiated alone (—) or irradiated and infected with AdMLP.apoptin encoding apoptin (- - -) or irradiated and with AdCMV.LacZ (•••) expressing β-galactosidase. The relative colony survival rate was calculated by setting the non-irradiated control-treated colonies at 100%, as described in the Materials and Methods section. The data points represent the average from two separate experiments. section. The data points represent the average from two separate experiments.

Apoptin sensitizes apoptosis pathways in radiation-treated SQD9 cells.

Besides the effect of combinatorial treatment of radiation and apoptin on the clonogenic cell survival, we also examined whether apoptin can sensitize the activation of apoptosis pathways in irradiated cells. Therefore, we investigated whether caspase-3 activity and cytochrome c release from the mitochondria could be detected after apoptin or β-galactosidase treatment in combination with irradiation. Both radiosensitive SCC61 and radioresistant SQD9 cells were infected with AdMLP.apoptin or AdCMV.LacZ and subsequently irradiated with 2 Gy, as described in the Materials and Methods section. Four days later, the activation of caspase-3 and cytochrome c release by apoptin and β-galactosidase were evaluated in parallel experiments by separating equal amounts of cytosolic protein on PAA-SDS and by means of Western blot analysis using specific antibodies directed against caspase-3 or cytochrome c, as described in the Materials and Methods section.

Radiosensitive SCC61 cells underwent caspase-3 activation as visualized by the appearance of the 17-kDa active subunit of caspase-3 and cytochrome c release after irradiation combined with apoptin or with β-galactosidase (Figure 3).

Figure 3

	SCC61		SQD9	
	apoptin & radiation	LacZ & radiation	apoptin & radiation	LacZ & radiation

A — Cytochrome c

B — Cleaved Caspase-3

Apoptin combined with irradiation triggers activation of caspase-3 and release of cytochrome c in radioresistant SQD9 cells. Irradiated SCC61 and SQD9 cells were infected with AdMLP. Apoptin or with the negative control AdCMV.LacZ. Panel A. Treated SCC61 and SQD9 analyzed by Western blotting for mitochondrial cytochrome *c* release. Panel B. Caspase-3 activation in treated SCC61 and SQD9 cells was analyzed by detection of cleaved 17-kDa active caspase-3.

In contrast, irradiation of the radioresistant SQD9 cells combined with β-galactosidase expression did not result in a detectable activation of caspase-3 (Figure 3A) or release of cytochrome c from the mitochondria (Figure 3B).

However, treatment of SQD9 cells with apoptin and irradiation resulted in a clear activation of caspase-3 (Figure 3A) and release of cytochrome c (Figure 3B).

These results clearly show that irradiation of the radioresistant SQD9 cells fail to efficiently induce apoptosis pathways, but these pathways are activated upon addition of apoptin synthesis resulting in an additive cytotoxic effect as observed in the described colony survival assays (Figure 2).

Discussion

Resistance of head and neck squamous cell carcinoma to conventional therapy, such as radiotherapy, is due to acquired deficiency in the apoptotic program[17]. The present *in-vitro* studies showed that the tumor-selective apoptosis inducing protein apoptin has a clear additive cytotoxic effect on human squamous carcinoma cells treated by irradiation.

By means of clonogenic survival assays, concurrent treatment of SQD9 radioresistant cells with irradiation and apoptin was proven to create an increase in apoptotic cell death in comparison to irradiation alone. The achieved cell death level reached almost comparable levels to those in radiosensitive SCC61 cells, which suggests that apoptin sensitizes the radioresistant SQD9 cells to irradiation. This assumption is strengthened by the findings that irradiation alone failed to activate the pro-apoptotic factors caspase-3 and mitochondrial cytochrome c release in the radioresistant SQD9 cells, but which occurred upon irradiation in combination with apoptin synthesis. These results suggest that in contrast to radiation, apoptin can activate the apoptotic machinery in SQD9 cells resulting in the release of mitochondrial cytochrome c and subsequent activation of caspase-3.

Caspases[18] play an essential role in programmed cell death, in which caspase-3 acts as one of the apoptosis executioners. It is widely accepted that cell death after apoptin treatment leads to caspase-3 activation[19]. Besides

this activation, apoptin-induced apoptosis results in cytochrome c release subsequent PARP-1 cleavage and Apaf-1 activation[20]. All these apoptotic processes that are activated by apoptin seem to be beneficial for enabling irradiation-induction apoptosis in SQD9 cells. However, thus far mechanistic aspects remain unknown.

Cancer cells typically evade apoptosis, partly due to an inhibitory mechanism on the mitochondrial cytochrome c release and subsequent activation of caspase-3. Irradiation alone did not induce cytochrome c release in the radioresistant SQD9 cells, whereas it did in the radiosensitive SCC61. What has been changed in the SQD9 cells and what do they share with SCC61 cells, for they are both sensitive to apoptin-induced apoptosis? Strikingly, apoptin can induce selectively apoptosis in a rather broad range of various types of cancer cells[21,22].

The exact mechanism of apoptin-induced apoptosis still remains unclear. The fact that apoptin induces apoptosis in radiosensitive and in radioresistant HNSCC cells to a similar extent, suggests that apoptin induces apoptosis in a different way than radiation does. Apoptin can induce apoptosis in the absence of the tumor suppressor p53[22,23], whereas it is known that radiation acts via active p53[24]. Recently, it has been shown that apoptin binds to the anaphase promoting complex (APC/C) with resulting cell cycle block in G2M and p53-independent cell death. This indicates that apoptin might be useful for the treatment of those tumors, which have lost p53 and are therefore resistant to many forms of anticancer agents[25,26]. In addition, various reports have shown that apoptin can induce apoptosis in cell lines expressing anti-apoptotic proteins such as survivin, Bcl-2, Bcl-xL and XIAP[16,27,28]. These circumstances are known to inhibit anticancer treatments such as radiation and chemotherapy[29,30]. All these features reveal that tumor-related blocks, within the apoptotic machinery, negatively affect conventional therapies but are not of relevance for apoptin-induced apoptosis. Further studies have to be carried out to unravel the precise mechanism of apoptin-induced apoptosis and its role in enhancing radiation-induced cell death.

The enhancement of radiotherapy with apoptin seems a meaningful option of further clinical investigation. Several studies have shown to increase survival and local control when patients are treated with the combination of chemotherapy and radiotherapy. The additive effect of concurrent therapeutic agents seems beneficial for a clinical outcome of head and neck cancer[31]. Besides our present studies on the beneficial effect of apoptin and radiation in radioresistant tumor cells, also other studies have reported the beneficial effect of a combinatorial treatment of apoptin with other therapeutic agents. Recently, Olijslagers et al. reported an additive effect of apoptin to the chemotherapeutic drugs etoposide and paclitaxel in human tumor cells, independent of p53[23]. In addition, Lian et al. analyzed the anti-tumor potential of simultaneous apoptin and interleukin-18 gene transfer in C57BL/6 mice bearing Lewis lung carcinoma[32]. They reported that the growth of established tumors in mice immunized with plasmid pAPOPTIN encoding apoptin in conjunction with plasmid pIL-18 encoding interleukin-18 was significantly inhibited as compared with the growth of tumors in mice immunized with plasmid pIL-18 or plasmid pAPOPTIN alone. Liu et al. have observed that addition of the acid ceramidase inhibitor LCL204, in combination with apoptin, augmented tumor-cell killing under *in vitro* conditions[28]. This effect was also demonstrated *in vivo* in that apoptin and LCL204 co-treatment significantly reduced tumor growth in DU145 xenografts. Our present and other studies demonstrate that apoptin is a promising therapeutic agent for various types of cancer and that its function is improved when combined with relevant anti-cancer age.

Conclusions

Our studies demonstrated for the first time that apoptin enhanced the radiosensitivity of human (head and neck) tumor cells. Concurrent irradiation and apoptin treatment harbors potential for being used as a therapeutic approach in overcoming radioresistance in treatment of HNSCC patients.

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