Apoptin gene therapy in head and neck cancer

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General Discussion

In this thesis, the mechanistic and therapy-related studies on apoptin tumor-selective apoptosis in head and neck squamous cell carcinoma cells *in-vitro* and *in-vivo* are described. Here, a brief summary mentions the various apoptin studies and the generation of a novel head and neck mouse tumor model. In the concluding remarks, a broader context of the potential of apoptin as a future therapeutic option for the treatment of head and neck squamous cell carcinoma is provided.

Summary

Head and neck cancer is one of the most common types of cancer accounting for 4 percent of all new cancer cases in the Netherlands[1]. The majority of these cancers are of the squamous cell carcinoma type. Primary treatment of head and neck cancer depends on the location of the tumor and stage at the time of diagnosis. Approximately 30% of all patients are diagnosed with advanced head and neck cancer, meaning loco-regional metastasis and/or stage T3 and T4[2]. In the last 2 decades, concurrent chemoradiotherapy and radiation for the treatment of loco-regionally advanced (stage III/IV) head and neck cancer has been widely studied[3]. The outcome of these studies has been organ preservation while maintaining or improving loco-regional control compared with radiotherapy alone and/or surgery. Secondly, it has resulted in improvement in absolute overall five year survival of 8%[3]. On the other hand, follow-up studies have indicated that side-effects of chemoradiation are considerable. In addition, in many cases the preserved organs were less functional than expected, leaving patients with considerable problems with eating, swallowing, voice and breathing. Furthermore, despite the improvement in disease-free and overall survival, up to 40% of all patients with head and neck cancer die due to this disease[4]. For stage III and IV treated with the combination of cisplatin/5-FU and radiation the reported 5-year outcomes overall survival is 39%[5].

Because this future perspective remains bleak from a patient perspective, new therapeutic alternatives, such as gene therapy, are investigated for the treatment of head and neck squamous cell carcinoma. Supported by a better understanding of the genetics of cancer[6], researchers try to produce drugs that eliminate tumor cells while sparing normal tissues[7]. Hereby there is a target-orientated approach aimed at genes whose products are the keystone for tumor expansion. Various strategies for gene therapy are considered, such as genes killing tumor cells directly, restore a defective tumor-suppressor gene, or induce apoptosis. Nearly all of these new options are still in preclinical development, such as HSV-TK/GCV, although some already passed the preclinical phase, as is the case with ONYX-015 and TNF–related apoptosis-inducing ligand (TRAIL). TRAIL is a member of the tumor necrosis factor α superfamily and induces cell death by the recruitment of caspase-8 and caspase-10 by the Fas-associated protein with death domain (FADD) and subsequential formation of the death-inducing signaling complex (DISC) and activation of caspase-3 and leading to the eventual cellular disassembly[8] and seems to be inactive against normal healthy tissue[9]. HSV-TK/GCV is based on the transfer of the herpes simplex virus thymidine kinase (HSV-TK) gene into tumor cells, which then selectively sensitize to ganciclovir (GCV) leading to an accumulation of its cytotoxic metabolite, GCV triphosphate (GCVTP), and subsequent incorporation into DNA and apoptosis[10]. HSV-TK/GCV is also termed suicide gene therapy and is made more effective by the phenomenon of the "bystander effect", which is the ability of suicide enzyme-expressing cells to sensitize neighboring non-expressing bystander cells to the pro-drug. The latter may be beneficial to surpass the principal hurdle in cancer gene therapy and to express the product in sufficient number of tumor cells. ONYX-015 can replicate in both tumor and non-cancer cells that lack functional p53, showing a cytopathic effect for tissues expressing an abnormal p53 gene, without damaging normal tissue and killing all kind of cells *in-vitro* such as cervical carcinoma cells, colon carcinoma cells, glioblastoma cells and pancreatic adenocarcinoma cells lacking functional p53[11]. Although there are

still uncertainties regarding the mechanism of ONYX-15 and the selectivity for p53-deficient cells and p53 as a target for anti-cancer drug development[12] remains unanswered. Several Phase I and II trials have, by means of intra-tumoral injection to deliver ONYX-015 into solid tumors shown that it is good tolerable at maximum administered dosages (10^{13} PFU) without reaching the dose-limiting toxicity (DLT)[13]. The most promising result came forward from a phase II study combining ONYX-015 with chemotherapy[14]. The here described protein apoptin is still in its preclinical phase although both *in-vitro* as *in-vivo* experiments are promising in part of the non-toxicity and anti-tumor effect[15-19]. Also the in this thesis described studies underline these observations found in various tumor systems and carried out by various research groups worldwide[19, 20].

Following a brief introduction on the current knowledge of gene therapy, and apoptin gene therapy in particular, the outline of this thesis is presented in **chapter 1**. The contemporary approach to the treatment of head and neck cancer with either (the combination of) surgery, irradiation and chemotherapy as well as their limitations are discussed. The need to further improve current therapy and the novel approach based on gene therapy are described. The chicken anemia virus-derived protein apoptin is a serious candidate for it induces cell death in tumor cells in a p53-independent manner. Especially, functional p53 is known to be required for mediating chemo- and radiotherapy induced apoptosis, but is in more than half of the tumors non-functional[19,21]. Several tumor cells derived from e.g. cervical cancer, prostate cancer and osteosarcoma undergo apoptosis once apoptin is expressed in these cells. Furthermore, over-expression of anti-apoptotic Bcl-2 does not hamper the effectiveness of apoptin. Nor, does the expression of the caspase inhibitors XIAP and CIAP or Bax, survivin, and FLIP(S) hamper apoptin- induced cell death[19]. These features are even further arguments that apoptin harbors potential novel anti-cancer activities.

In **chapter 2,** a study is described in which a cell line derived from poorly differentiated squamous cell carcinoma of the floor of the mouth with a mutated non-functional p53 is treated with apoptin. Apoptin treatment resulted in the activation of the apoptosis executioner caspase-3 and the release of mitochondrial pro-apoptotic cytochrome *c*. In these cells, both p53 and apoptin could effectively induce apoptosis, reaching an apoptosis rate of approximately 80% after 4 days. When Bcl-xL was co-expressed with p53 or apoptin in these squamous cell carcinoma cells it clearly affected the apoptosis rate of p53, but not the rate of apoptin-induced apoptosis. In the presence of Bcl-xL, apoptin can still induce apoptosis in the majority of the cells producing both Bcl-xL and apoptin. In accordance Bcl-xL diminished the p53-induced cytochrome c release, but not the apoptin activated one. Radiation is widely used in the treatment of squamous head and neck carcinomas and causes damaging of DNA in the rapid dividing tumor cells, which results in apoptosis induction via p53-mediation, though this can be blocked by Bcl-2 anti-apoptosis members such as Bcl-xL[21]. In attempts to overcome the resistance, higher doses of radiation were used with consequential bystander damage to normal tissues and an undesirable degree of toxicity.

In **chapter 3,** the combination of apoptin and radiation was investigated *in-vitro* in two cell lines. One radiosensitive and one more radioresistant human head and neck squamous cell line were examined. By adding apoptin before treating the cell lines with radiation the amount of cells becoming extinct increased considerably. Both cytochrome c release and caspase-3 cleavage could be detected in radioresistant cells when apoptin was added to irradiation treatment, whereas irradiation treatment alone did not. By means of colony survival assays it was proven that a combinatorial treatment of apoptin and irradiation revealed a beneficial effect on cell death in comparison to single treatment with apoptin alone. These results are in accordance with *in-vitro* or *in-vivo* studies carried out by others[22-24]. They showed that combined treatments of apoptin with (chemo)therapeutic agents such as paclitaxel, IL-18 or acid-ceramidase inhibitor LCL-204 resulted in a more potent anti-tumor effect than the single treatments.

In **chapter 4** several alternatives for investigation of new treatment options such as gene therapy are assessed. *In-vitro* cell lines, xenografts in immunodeficient mouse and the hamster cheek pouch model in which 7,12-dimethlbenz(a)anthrance is used to induce tumors, have all their limitations. The carcinogen 4-nitroquinoline-1-oxide (4NQO) is known to induce squamous cell carcinomas of the palate and the tongue in rats and mouse after repetitive oral application. In our studies, 4NQO was applied three times a week during 16 weeks in male CBA mouse. The time that was needed for representative squamous cell carcinoma with an appropriate volume for tumor treatment was proven to be another 24 weeks. The established squamous cell carcinomas and the preceding dysplastic changes were evaluated and compared by using the immunohistochemistry tumor markers cyclin D1 and E-cadherin. These immunohistochemistry studies revealed that the obtained 4NQO-induced primary mouse oral tumors showed strong similarities with the described squamous cell carcinomas in humans. Therefore, this model is suitable for conducting pre-clinical research in oral squamous cell carcinoma starting from approximately 40 weeks after the start of the experiment. The prior mentioned pre-malignant oral dysplastic lesions are a separate entity with their own complexity of diagnosis, treatment and follow-up.

In **chapter 5** we have taken a closer look at the oral dysplasia in the 4NQO model in the mouse and try to determine if the model can be valuable for laboratory investigations. During sixteen weeks, 4NQO treatment in the mouth of male CBA mouse was carried out and at several time points afterwards developing oral dysplasia was observed. Several architectural and cytological characteristics essential in human pathology were used to describe the possible dysplastic changes. Microscopic assessment of the mucosa of the mouse tongue revealed that all three consecutive stages of epithelial dysplasia, mild, moderate and severe dysplasia were seen. All three stages contained architectural and cytological features seen in human dysplasia, which implies that the carcinogenic mouse model resembles that

of the human counterpart, making it apt for laboratory investigation. The time point when mild, moderate and severe dysplasia is encountered became apparent in our studies, which will make future research on these primary tumors more convenient.

In **chapter** 6 the *in-vivo* effect of apoptin is assessed in the 4NQO model. By applying the 4NQO carcinogen in immunocompetent male CBA mice, tongue tumors were induced which were suitable for treatment and were injected with a single dose of a recombinant adenoviral vector expressing apoptin (AdMLP.apoptin). A considerable amount of tumor cells expressed apoptin and underwent apoptosis, whereas injection with the negative control AdCMV.LacZ expressing β-galactosidase did not. In parallel experiments, squamous cell carcinoma cells were derived from the primary tongue tumors and grown in culture. Passage 1 cells were treated with apoptin and the result was a considerable amount of cells undergoing apoptosis. Both the *in-vivo* and *in-vitro* apoptin-treated cells underwent apoptosis via the activation of caspase-3. The fact that apoptin induces apoptosis in primary squamous cell carcinoma cells indicates that apoptin is a potential therapeutic agent for treatment of head and neck squamous cell carcinoma.

Concluding Remarks

The protein apoptin is derived from the chicken anemia virus was discovered by and first described by Noteborn et al. in 1994[25]. Since then, many surprising results have been obtained in our laboratory and in various other research institutes worldwide. A vast range of different tumor cell lines undergo apoptosis after apoptin treatment, whereas normal cells are not affected by apoptin. In this thesis, 4 different types of squamous carcinoma cell lines are treated with apoptin resulting in cell death in all cases. The apoptin-sensitive UMSSC-14B cell line contains non-functional p53. Even co-synthesis of Bcl-xL in these tumor cells did not hamper apoptin induced cytochrome c release. Release of cytochrome *c* from mitochondria is a central event in the death receptor-independent, "intrinsic", apoptotic pathway, which results with the help of ATP and Apaf-1 in the activation of caspase 9 and downstream effector caspases, which then cleave their substrates, finally leading to the apoptotic cell death. In addition, cytochrome *c* release also occurs in death receptor-dependent, "extrinsic," apoptotic pathways by cleavage and activation of the pro-apoptotic Bcl-2 family member Bid through caspase 8, possibly serving as an amplification loop.

The observations that despite the lack of functional p53, apoptin was able to induce the release of mitochondrial cytochrome *c* in UMSCC-14B cells, even when Bcl-xL was co-produced, are very interesting ones. Co-synthesis of p53 and Bcl-xL in UMSCC-14B cells did not resulted in a cytochrome c release. In addition, the radioresistant head and neck squamous carcinoma cell line SQD9 cells became sensitive to irradiation upon co-treatment with apoptin and there was a clear additive effect seen in cell death. Irradiation alone did not result in release of cytochrome *c* or activation of caspase-3. In chemo- and radioresistant cells an up-regulation of Bcl-xL is known to block the chemo-/radiation-induced apoptotic signal by inhibiting the mitochondrial release of cytochrome *c* and activation of both upstream and downstream caspases[26]. These data convincingly show that apoptin can induce apoptosis

under conditions, when chemo- or radiotherapy will fail. Earlier reports demonstrated that baculovirus derived p35, which inhibits caspases, does not inhibit the release of cytochrome c after apoptin treatment[27]. CrmA, which is derived from the cowpox virus, is known to block the upstream caspases 1 and 8, although it does not hamper the effectiveness of apoptin. Caspase-3 is the main downstream effector caspase and is activated just prior to the apoptosis when it cleaves the majority of the cellular substrates. All cell lines, whether radiosensitive or more radioresistant or passage 1 cells derived from a primary tongue tumor from a mouse, possessed active caspase-3 upon apoptin treatment. In the animal model, caspase-3 was detected 2 days after treatment with apoptin. As a whole activation of the executioner caspase-3 appears to be pivotal for apoptin induced apoptosis. The remarkable fact that apoptin only induces apoptosis in transformed and not in normal cells appears to lie in the fact that apoptin harbors a nuclear localization in tumor cells, while in normal cells it is located in the cytoplasm. Furthermore, nuclear apoptin is phosphorylated in tumor cells though unphosphorylated in normal cells. This phosphorylation of apoptin at the threonine 108 site appeared to be tumor selective and enables apoptin to accumulate in the nucleus resulting in cell death[28]. Because nuclear localization seems crucial for apoptin activated cell death, we hypothesize that a signal has to go from the nucleus to the mitochondria and the downstream caspases. Further studies are needed to unravel the precise mechanism of apoptin's tumor-selective apoptosis capabilities.

As with other types of gene therapy, apoptin gene therapy has its limitations. Even though there has been significant progress achieved in vector technology, effective transfer of genes *in-vivo* remains elusive. When delivered systemically tumor cells are not targeted specifically. As with other types of cancer, head and neck cancer frequently metastasizes so direct delivery into the (primary) tumor maybe insufficient. In the *in-vivo* experiments we noticed that a fast amount of cells were expressing apoptin

after treatment, but in order for gene therapy to be successful it is necessary to deliver the gene in every cell or the remaining nontransduced tumor cells will continue to proliferate. There is not an ideal vector and each one has its specific limitations. At present viruses are the most commonly used vectors because of their (relative) high efficiency of transduction. Of these the adenovirus seems to be the most promising of all viruses. Currently viral vectors are evaluated that are genetically engineered for tumor selectivity by either modifying the cellular tropism at the level of viral replication in a way that it becomes dependent on specific characteristics of tumor cells for viral replication or enhancing the tumor-selective binding and uptake of the vector. Of the non-viral vectors several peptides have shown the ability to deliver proteins across the cell membrane[29, 30]. Of particular interest is the positively charged domain found in the HIV-1 trans-activator protein TAT, the TAT protein transduction domain (TAT-PTD). The TAT-fusion proteins seem to transduce cells efficiently and *in-vivo* studies in mice have shown that after intraperitoneal injection of TAT-β-galactosidase enzymatic activity could be detected in all tissues tested, including the brain[31]. Recent results with TAT-apoptin protein showed that TAT-apoptin efficiently killed tumor cells[32], while sparing non-malignant human cells[33]. These new findings are encouraging and increase the possibility of apoptin being used in cancer (gene) therapy. Recently, Sun et al. have shown that PTD4-apoptin protein induces apoptosis in xenografted human tumors established in nude mice models, but leaves normal cells unharmed[34]. In the near future the success of cancer gene therapy will dependent on the improvement of its vectors. Until then gene therapy must be combined with present therapies, such as surgery, irradiation and chemotherapy, to decrease the number of cancer cells in the initial treatment

Summarizing, this thesis describes in *in-vitro* and *in-vivo* experiments the potential of apoptin as a future drug in the treatment of head and neck squamous cell carcinoma. Reliable and representative animal models were composed and evaluated based on human pathology characteristics describing the degree of dysplastic changes and squamous cell carcinoma. The *in-vitro* results show that apoptin effectively induces cell death in a p53-independent way, is not hampered by up-regulation of anti-apoptotic Bcl-xL and able to kill radioresistant head and neck tumor cells. A single intra-tumoral injection of a recombinant adenoviral vector expressing apoptin resulted in considerable cell death and cells underwent apoptosis by activation of caspase-3. These results are encouraging and apoptin may constitute a potential therapeutic agent for treatment of head and neck cancer.

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