Apoptin gene therapy in head and neck cancer

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Introduction and outline of this thesis

Head and neck squamous cell carcinoma (HNSCC) represents the fifth most common cancer in the world, with a global incidence of more than half a million each year[1]. In the Netherlands approximately 2650 patients are diagnosed with head and neck cancer each year, which accounts for approximately 4% of all new malignancies per year[2]. The main risk factors for developing HNSCC are tobacco and alcohol consumption[3,4]. In the Netherlands the incidence of males with a head and neck malignancy is much higher than that of females with a ratio of 1 to 5.5. This can be attributed to smoking and drinking custom of males that is compared to females much higher. Although the 5-year survival rate for head and neck cancer has reached approximately 50%, it is one of the lowest of the main cancers[5]. Part of this is due to the fact that more than 50 percent of the patients are diagnosed with advanced stages at diagnosis. Surgery and radiotherapy are the two main therapeutic preferences, although concurrent and/or adjuvant chemotherapy is elaborately being investigated and plays an increasing and promising role in the primary treatment with organ preservation in advanced stages of head and neck cancer. Recent studies revealed that concurrent postoperative chemotherapy and radiotherapy significantly improve disease-free survival although the combination of chemotherapy and radiation is associated with a substantial increase in adverse effects[6]. Still, local or regional disease recurs in 30% of patients and 25% develop distant metastases[7].

Notwithstanding the fact that current treatment outcome has improved, there is still room for new experimental therapeutic options, such as gene therapy[8].

Gene therapy

Gene therapy is the delivery of genes into a cell or tissues in order to treat a disease in which a defective mutant allele is replaced with a functional one and resulting in either curing the disease or slowing down the progression of it. These diseases can be either acquired or inherited. In diseases such as

cancer, gene therapy is used to either directly or indirectly cause the demise of the cancer cells. In other inherited diseases, gene therapy is used to introduce missing genes into the patient. Current diseases that are being investigated for gene therapy are cancer, cardiovascular disease, neurodegenerative disorders such as Parkinson's and Alzheimer and infectious diseases such as HIV. Nowadays, it is commonly acknowledged that gene therapy has got great potential for the treatment of cancer[9]. The idea of gene therapy was first proposed during the fifties[10] and pioneered during the seventies and eighties and the first approved gene therapy method was performed on a young patient suffering from severe combined immunodeficiency (SCID). Since then numerous research has been done and trials have been undertaken with gene therapy[11]. Gene therapy seems logical, especially in diseases with a single gene defect such as cystic fibrosis, haemophilia and deafness. To realize this, gene therapy requires technologies capable of gene transfer into all kind of cells, tissues, and organs, a so called vector. The vector is the vehicle that carries the genetic information into the targeted cell. At this time, the most frequent used vector is a virus that has been genetically altered to carry normal human DNA. Viruses are capable of introducing their genetic material into host cells and further produce copies of these viruses and eventually infecting more cells. By manipulating the virus and removing the disease-causing genes and/or inserting therapeutic genes one can take advantage of this capability.

The predominant obstacle in the way of successful application of gene therapy is the improvement of safe and effective vectors to transfer the DNA into a cell[12] and is still the Achilles heel of gene therapy. After some early promising results by pioneers in gene therapy an 18 year old volunteer died while participating in a clinical trial and became the first known victim of gene therapy. This caused wide spread controversy and although the cause of death was not linked to the viral vector, nowadays four basic requirements are set for the viral vector. These are safety, low toxicity, stability and cell-type specificity. The properties of an ideal vector are furthermore: easy to

produce, should be able to express its genetic cargo over a sustained period or expression, immunologically inert, meaning that it should not elicit an immune response after delivery, preferable tissue specific, the vector should have a considerable size that it can deliver, must infect both dividing and non-dividing cells[13] and must have a site-specific integration of the gene into the chromosome of the target cell. The latter though is not necessary in cancer gene therapy. Vectors can be divided into two categories, non-viral such as plasmids and liposomes and viral vectors such as retroviral, adenoviral, and adeno-associated viral vectors. Non-viral vectors have the disadvantage that they have an inefficient gene transfer and the expression of the incorporated gene is transient, although they can be easier produced in relatively large amounts, and present fewer toxic or immunological problems. Viral vectors give a sustained and most of the times a high-level expression of the gene and right now are the most suitable vehicles available, although none of the earlier mentioned characteristics can be found in one single vector. The features of the most common vectors are described in table 1.

Apoptosis and cancer

Apoptosis is a securely regulated and complex procedure to deliberately kill a cell and together with autophagocytosis it is the other form of programmed cell death[14]. Apoptosis occurs when a cell is damaged beyond repair, experiences stress, is infected with a virus or when its DNA is damaged by radiation or chemotoxic agent. When this intrinsic mechanism of self destruction fails, leading to too much growth and too little cell death, cancer is the ultimate result. A defect in this mechanism can not only lead to expansion of tumor cells, but because conventional therapies such as chemotherapy and irradiation act primarily by inducing apoptosis, a non-functional apoptotic pathway can result in cancer cells becoming more likely to be resistant to therapy. A key player in regulating apoptosis is the tumor suppressor p53 protein, often called the “guardian of the genome”[15]. p53 is activated upon

Table 1 Properties of vectors

Vector	Agent	Packaging capacity	Integration of genome	Immunogenicity	Limitations	Advantages
Non-viral	Naked DNA	> 10 Kb	No	Variable	Variable gene expression	Higher expression with the use of *'gene-gun'*
	Liposome	> 10 Kb	Episome	Low to moderate	Low transduction efficiency	Lack of immunogenicity
Viral	Adenovirus	8-30 Kb	Yes	Variable	Capsid causes potent inflamation	Extreme efficiency of transduction
	Adeno-associated virus	<5 Kb	Yes	Low	Small packaging capacity	Non inflammatory
	Retrovirus	9 Kb	Yes	Low	Only transduces in dividing cells	Persistent gene transfer in dividing cells
	Lentivirus	<9 Kb	Yes	Low	Integration can induce oncogenesis	Persistent gene transfer in most tissues
	Herpes simplex virus	40-150 Kb	Extra chromosomal	Low	Inflammatory	Large packaging capacity

DNA damage, stress or proliferative signals and will either lead to cell cycle arrest for repair or apoptosis. Loss of p53 leads to genomic instability, impaired cell cycle regulation, and inhibition of apoptosis. There are two pathways that initiate apoptosis, the extrinsic pathway[16] that is mediated by death receptors on the cell surface and the intrinsic pathway[17] that is mediated by mitochondria. Death receptors such as CD95, TRAIL-R1 or TRAIL-R2 are activated in response to ligand binding and initiate caspase-8 and caspase-10. Activation of mitochondria is mediated by the Bcl-2 family and results in the release of cytochrome c in the intermembraneous space of mitochondria and the cytosol[18]. Consequently cytochrome c forms a complex with Apaf-1 and the inactive initiator caspase procaspase-9, now called the apoptosome resulting in caspase-9 activation.

Apoptosis can be regulated by various proteins that regulate this process at different levels. First, there are the members of the Bcl-2 family, which regulate apoptosis at the mitochondrial level[19]. According to their function they can be divided into anti-apoptotic and pro-apoptotic proteins. Bcl-2 family proteins influence the permeability of the mitochondrial membrane and can be divided in pro-apoptotic (Bax and BAD among others) or anti-apoptotic (including Bcl-2 and Bcl-xL)[20]. Secondly, the IAPs (inhibitor of apoptosis proteins) bind to and inhibit caspases[21]. So far nine IAP family members have been identified and IAPs are inhibited by SMAC/DIABLO, which is released from mitochondria upon apoptosis along with cytochrome c[22, 23]. Thirdly, FLIPs can directly interfere at the level of death receptors[24]. Finally the downstream caspases-3, -6 and -7 are the 'executioner' caspases and upon activation they cleave each other and cleave cellular death substrates leading to characteristic biochemical and morphological changes[25].

Cancer treatment such as irradiation and chemotherapy kills cells primarily by the initiation of apoptosis. Nevertheless, not many tumors are sensitive to these therapies, and patients developing a relapse of a tumor usually have tumors that are more resistant to therapy than the primary tumor. The latter can be due to apoptotic defects with consequential multidrug

resistance[26]. Additionally the mitochondria-dependent pathway is frequently hampered, by over-expression of Bcl-2 or Bcl-xL[27]. Down-regulating Bcl-2 and Bcl-xL with antisense techniques enhances cellular response to chemotherapy[28, 29]. Several studies have demonstrated that over-expression of Bcl-2 in cancers is linked with reduced response to chemotherapy[30, 31].

Apoptin

A number of promising viral and cellular proteins are known to launch apoptosis in cancer cells, such as TRAIL[32], a member of the tumor necrosis factor (TNF) family, mda-7[33] (melanoma differentiation-associated gene-7), E4orf4[34] (Adenovirus early region 4 open reading frame 4) and apoptin[35, 36]. Apoptin the focus of this thesis is a 13.6-kDa chicken anemia virus derived protein of 121 amino acids[37]. The chicken anemia virus contains a single stranded genome encoding three partially overlapping proteins, VP1, VP2 and VP3 (apoptin). Apoptin induces apoptosis in a great variety of human tumor cells[38] and remarkably not in a range of normal human cells[39]. Cell death due to apoptin is p53 independent[39, 40] and tumor selective. Subsequent studies showed that adenovirus-mediated transfer of apoptin into normal cells is not toxic for these normal cells *in-vivo*[39]. The latter is being attributed due to the fact that apoptin has a nuclear localization in tumor cells and quite the opposite in the cytoplasm of normal cells[41]. Apoptin is phosphorylated in tumor cells and in contrast in normal unphosphorylated[42, 43]. *In-vitro* research in a vast range of tumor cell lines e.g. derived cells from osteosarcoma[44], hepatoma[45, 46] and lymphoblastoma revealed that apoptosis upon apoptin activates cytochrome c[46] and is insensitive to Bcl-2[47]. Breast adenocarcinoma cell lines treated with apoptin revealed that Nur77 translocates from the nucleus to the cytoplasm and transmits an apoptotic signal to the mitochondria[48]. Additionally the *in-vivo* effect of apoptin in animal models revealed that single injection of apoptin transmitted by an adenoviral vector resulted in a delay of tumor growth in xenografted hepatomas in nude mice[45]. Treatment

with fowlpox virus expressing the apoptin protein in subcutaneous growing hepatoma in mice resulted in a strong antitumor response and a reduction in tumor size[49].

Models to study gene therapy

Experimental models enable us to test potential therapies prior to human trials. *In-vitro* and *in-vivo* procedures each have their strengths and limitations. Culture cell research in dishes has the advantage of stringent controlled circumstances, direct visualization and direct accessibility. The limitation of *in-vitro* investigation is that artificial components in the culture may cause different cellular responses to what might occur *in-vivo*. This may reflect the loss of cellular features normally expressed *in-vivo* and non-physiological response to experimental treatments due to lack of 'normal' circumstances. Although *in-vitro* testing is considered the standard first step for assessing the significance, the results might not be clinically applicable. *In-vivo* studies occur in animals and advantageous are the physiological responses, the use of entire organs and the replication of the disease. Nude mice when injected with xenografts of human tumors reproduce comparative histological pattern of most human tumors, although they lack a regular physiological reaction. Limitations of animal models are the costs, the frequent anesthesia that must be used and the certain amount of unpredictability compared with cell cultures.

Aim and outline of this thesis

Because of the disappointing progress that has been made in the last decades in survival in patients with head and neck cancer, existing therapy needs to be improved and/or new treatment needs to be introduced. This thesis describes a new promising treatment, apoptin gene therapy. The scope of this study was to investigate the applicability of apoptin in head and neck

squamous cell carcinoma (HNSCC). First, we assessed its potential *in-vitro*. Next, a suitable animal model was established, which was used for *in-vivo* experiments with apoptin.

In **chapter 2** we describe the results of apoptin treatment in a HNSCC cell line with a mutated p53 and the effect of over-expression of Bcl-xL on the outcome. **Chapter 3** describes the synergistic effect of apoptin and irradiation in HNSCC both in radiation sensitive and in more radioresistant HNSCC cell lines. The applicability of an immune competent animal model for *in-vivo* research is described in **chapter 4**. The time needed to establish a useful oral squamous cell carcinoma in mice is assessed and immunological comparisons are made with human counterparts. In **chapter 5** the tumorigenesis of the same carcinogenic immune competent model is investigated and characteristics are analyzed. The efficacy of the apoptin therapy *in-vivo* is described in **chapter 6**. This is done by looking into the effect of intratumoral injection of a constructed adenovirus expressing the apoptin protein. Finally, the data are critically discussed in **chapter 7** in view of apoptin as a potential new anti-cancer therapy.

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