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1. Gene therapy

1.1 Definition of gene therapy
According to the European Medicines Agency (EMA), a gene therapy medicinal product means “a biological medicinal product that contains an active substance, which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view of regulating, replacing, adding or deleting a genetic sequence, as well as if its therapeutic, prophylactic or diagnostic effect relates to the recombinant nucleic acid sequence it contains, or to the products of genetic expression of this sequence”. Simplified, in the current meaning gene therapy is the transfer of nucleic acids to somatic cells of a patient to result in a therapeutic effect. Generally, gene therapy can be classified into two categories - somatic gene therapy and germ line gene therapy. The difference between these two approaches is that in somatic gene therapy the genetic material is inserted to selected target cells, but the genetic information is not passed along to the next generation, whereas in germ line gene therapy the genetic information is passed on to the next generation. The classification of gene therapy into these two categories is of importance, since as up to day legislation allows gene therapy only to somatic cells.

1.2 History of gene therapy
In 1952, Zinder and Lederberg introduced a new term “transduction” as to describe the transfer of genetic material to cells. They demonstrated that a phage (i.e. a virus that was referred to as a filterable agent) of Salmonella typhimurium can carry DNA from one bacterium to another (Zinder & Lederberg, 1952). Furthermore, they pointed out that the active “filtrate” (i.e. phage) could transfer (i.e. transduce) many hereditary traits from one bacteria strain to another strain. This was a very interesting and important finding, as it explained how bacteria of different species could gain resistance to the same antibiotic very quickly. This basic understanding that phages could transfer genetic materials was soon extended also to eukaryotic viruses and a decade later after Zinder and Lederbergs discovery, Howard Temin found that specific genetic mutations as a result of virus infection could be inherited (Temin, 1961). He observed in his experiments that chicken cells infected with the Rous sarcoma virus (RSV) stably inherited viral specific gene mutations that contained the information for the generation of RSV progenies. As in the case of Zinder’s
and Lederberg’s study, this observation was of great importance, as it unveiled the conundrum that genetic information would flow only from DNA to RNA. As the Rous sarcoma virus is a RNA virus, Temin’s study showed that information could also flow from RNA to DNA, which subsequently led to the discovery of RNA-dependent DNA polymerases. Furthermore, it was realized that the acquisition of the new characteristic was stably inherited and that this was through the chromosomal insertion of the foreign genetic material (Sambrook et al, 1968).

It was obvious that viruses had properties that could be very useful in delivering genes into cells of interest. Accumulating evidences of successful cell transformation studies gave rise to the thought that genetic engineering may become a novel means for treating genetic diseases. In 1966, Edward Tatum published a paper arguing that viruses could effectively be used for modulating somatic cells and hence be a intriguing tool for gene therapy (Tatum, 1966). Of course, it was also clear that it would be necessary to strip those viruses from their pathology causing genes and replace them with a therapeutic gene or genes. Unfortunately, at that time appropriate tools for recombinant DNA technologies were not established yet. However, this did not prevent some scientists to continue working with viruses. A couple of years later after Edward Tatum’s paper, Rogers et al. demonstrated an initial proof-of-concept of virus mediated gene transfer. In this study, they provided proof that a transfer of a foreign genetic material is possible by using viruses. Rogers et al. used the tobacco mosaic virus as a vector vehicle to introduce a polyadenylate stretch to the viral RNA (Rogers & Pfuderer, 1968). Motivated by the results and the hypothesis that viruses might be useful tools to introduce genetic material to cells, they went even further. Several years later, the same group performed the first direct human gene therapy trial. In that study, they used the wild-type Shope papilloma virus with the intention to introduce the gene for arginase into two girls suffering from a urea cycle disorder (Terheggen et al, 1975, Rogers et al, 1973). Based on their previous studies, they believed that the Shope papilloma virus encoded the gene for arginase activity and that this gene could be transferred by introducing the virus to the patients. Unfortunately, the outcome of the trial was negative. There was no change, neither in the arginine levels, nor in the clinical course of the hyperargininemias. Later on, after sequencing of the virus genome, it turned out that the Shope papilloma virus genome does not encode an arginase.

The study by Rogers et al. already initiated discussions about the ethical aspects and rationality of gene therapy. But it was at the latest, when Martin Cline performed a study to treat thalassaemia patients, when a big ethical debate was initiated. In that study, bone marrow cells from thalassaemia patients were taken and transfected ex vivo with plasmids containing the human globulin gene, after which these cells were re-introduced to the patients (Beutler, 2001, MacMillan, 1982). The problem, however, was that Cline had not received permission to perform those studies from the UCLA Institutional Review Board, as well as there were clear concerns about the efficacy of this therapy, since earlier animal studies demonstrated negative results (Beutler, 2001, Mercola et al, 1980). As a result, it became clear that human gene therapy would be technically, as well as ethically more complex than it was expected. Serious technical hurdles to effective gene therapy were apparent. Firstly, since of the lack of appropriate tools for recombinant DNA technology, there was no means to obtain specific functional genes. Secondly, there was no efficient or targeted means by which to deliver or replace a non-functional gene with a functional one to a defective cell. It became clear that in order to have effective therapy, the functional gene
needed to be delivered to the right place and in a sufficient amount. Nevertheless, as compared to traditional medicine, gene therapy offered unique possibilities. The discovery of the existence of integrated proviruses of viral RNA genome and the reverse transcriptase lead to the development of new and more efficient retroviral vectors. This was expected to overcome some of the barriers of chemical transfection. Several experiments proved that retroviral vectors could be used to complement genetic defects and correct disease phenotype in human cell culture models. They suggested that many human cell types are amenable to retrovirus mediated gene transduction (Goncalves, 2005). The development of adenoviral vectors further improved transduction efficiency. In comparison to retroviral vectors, adenoviruses are able to transduce both dividing and non-dividing cells, however, resulting in a more transient expression profile. The initial targets for gene therapy were mainly monogenic disorders such as enzyme defects associated with metabolic diseases; namely Lesch-Nyhan syndrome, adenosine deaminase (ADA) deficiency, familial hyper-cholesterolaemia of low density lipoprotein (LDL) receptor deficiency, α1-antitrypsin deficiency, clotting factor deficiencies and Gaucher disease. Indeed, excellent results were obtained from ADA deficiency and adrenoleukodystrophy treatments with retroviral vectors (Aiuti et al, 2009, Cartier et al, 2009), but also failures were reported. One of the best known cases is the one of Jesse Gelsinger in 1999. Gelsinger suffered from a partial deficiency of ornithine transcarbamylase (OTC), a liver enzyme that is required for the removal of excessive nitrogen from amino acids and proteins. He was treated with adenoviral vectors encoding for the ornithine transcarbamylase gene. Four days after the treatment Jesse Gelsinger died from multiorgan failure. He was the first patient in whom death could be directly attributed to the viral vector used for treatment. From the inception of gene therapy, cancer has been a major target. It is by far the most common disease area where gene therapy is applied to, composing over 60% of all ongoing clinical gene therapy trials worldwide, followed by cardiovascular diseases and making up close to 10% of all trials (Figure 1). The first clinical trial on cancer started in 1990, where patients with advanced melanoma were treated with tumour infiltrating lymphocytes genetically modified ex-vivo to express tumour necrosis factor (Rosenberg et al, 1990). Since then, many cancer types have been targeted with gene therapy including brain, lung, breast, pancreatic, liver, colorectal, prostate, bladder, head and neck, ovarian and renal cancer (Figure 1) (Wirth et al, 2009). Up to year 2010, more than 1700 approved gene therapy clinical trials worldwide have been conducted or are still ongoing, but so far neither the Food and Drug Administration (FDA), nor the EMA have approved any human gene therapy product for commercial use. One of the major hurdles has been how to get the relevant genetic material into a sufficient number of target cells, and how to avoid the transduction of non-target cells (i.e. how to target the gene transfer vector to cells of interest)?

2. Gene transfer methods

Different methods for gene delivery have been studied and used. They can be grouped into three different categories: 1) physical, 2) viral and 3) non-viral methods. Examples of physical methods are electroporation, ultrasound and gene gun delivery. In the viral or non-viral gene transfer methods, a biological (a virus) or a synthetic (liposomes or nanoparticles) vector is used as a vehicle to deliver the genetic material into the cells. In the clinical trials the most commonly used gene transfer vectors have been adenovirus, retrovirus and naked/plasmid DNA (Figure 2). Even though lentiviral vectors represent currently only a
small percentage of the viral vectors being used, they have been intensively studied and may be a promising candidate in future gene therapy strategies.

Fig. 1. The graph shows the different indications that have been addressed by gene therapy in clinical trials. Even though initial studies have been conducted on monogenetic diseases, cancer became soon after a major interest, with up to date consisting 65% of all clinical trials. The reasons for this are the highly unmet medical need in cancer therapy, as well as its big market size. Also, the ethical acceptance of gene therapy as a therapeutic modality is a factor that surely has supported the shift from monogenetic diseases to cancer.

There are three main methods that have been utilized to deliver a genetic material into the brain: 1) Stereotactic intracerebral inoculation by craniotomy, 2) intrathecal or intraventricular administration and 3) intravascular administration of the gene transfer vector. Of those three methods, the stereotactic inoculation by craniotomy is the most commonly used strategy (Wirth & Yla-Herttuala, 2006). Genetic material can be delivered also by ex-vivo gene transfer approach, in which gene transduction is performed outside the patient (i.e. ex vivo), into previously isolated autologous cells, which are re-introduced back to the patient. Even though the intracerebral injection of gene transfer vectors is the simplest approach for local gene therapy, this method still faces obstacles that remain to overcome. The direct intraparenchymal injection is limited by the small volumes that can be administered into focal areas. Also, the diffusion of the gene transfer vectors is minimal, i.e. they do not significantly penetrate into brain parenchyma, which may restrict the transduction to only a few micrometers from the injection site (Rainov & Kramm, 2001). Convection enhanced delivery is a method to improve the spread of the gene transfer vector within the tissue by maintaining a pressure gradient during interstitial infusion (Bidros et al, 2010). In this method multiple catheters are placed within the tumour and the vector solution is infused at a continuous and slow rate (Bidros et al, 2010). However, there is still discussion about, whether convection enhanced delivery really improves transduction efficiency (Bidros et al, 2010, ter Horst et al, 2006). One of the limitations of direct intratumoural administration is that it is possible to do only with solid tumours. In case of
systemic administration of the gene transfer vector, targeting of the vector specifically to the tumour cells or restricting the expression of the transgene to tumour cells is essential (Bourbeau et al, 2007).

Viral vectors are considered as the most effective of all gene delivery methods for in-vivo gene transfer. Commonly used viral vectors for brain cancer gene therapy includes retrovirus, herpes simplex virus, adenovirus and adeno-associated virus (Figure 2). Apart from these, baculovirus, New Castles’ Disease virus, polio virus, semliki forest virus, measles virus, lentivirus, reovirus and vaccinia virus have also been used as vectors. In most of these vectors, the viral genome has been genetically modified to make them replication defective and non-pathogenic and to make space for the transgene. However, in the oncolytic viruses the viral genome has been modified to give them the ability to specifically replicate only within tumour cells that would lead to the lyses of the tumour cells. Some of the new viral vectors have the ability to replicate only once within the target cells without the ability to disseminate, thereby aiming at increasing the transgene copy number and expression within the transduced cells.

2.1 Adenoviruses as a gene transfer vector
Adenoviral vectors have been widely utilized for gene therapy studies both in preclinical and clinical settings. To date, more than 100 adenovirus serotypes have been classified, of which 51 serotypes are human adenoviruses, divided into seven species (human adenovirus A to F). These classifications are based on their hemagglutination properties, oncogenic potential in newborn hamsters, genomic organization and DNA homology (Sharma et al, 2009). Adenoviruses are non-enveloped, icosahedral viruses of 80 to 1200 nm in diameter.
They contain a linear, double-stranded DNA genome of 30 to 40 kb. As gene transfer vectors, they can carry relatively large fragments of foreign DNA. The main components of adenoviruses are the capsid and the core. The homotrimeric hexon capsomers, which are the main constituent of the capsid, form the 20 triangular faces of the icosahedron. Each of the 12 vertices on the adenovirus surface is made up of a penton capsomere. The penton capsomere is composed of a penton base and a fiber protein. The latter forms a spike-shaped protrusion with a terminal globular domain or “knob” that is responsible for the attachment of the virus to its primary receptor. In addition to hexon and penton capsomers, there are several hexon-associated proteins with capsid-stabilizing functions. The double-stranded DNA genome is located in the virion core with DNA-associated proteins and terminal proteins that serve as a primer for DNA replication.

Human adenoviruses are known to cause a variety of clinical symptoms, depending on their serotypes (Sharma et al, 2009). These include infections of the upper and lower respiratory tract, renal and urinary tract, gastroenteritis and ocular infections. The coxsackie-adenovirus receptor is a 46 kDa type I transmembrane glycoprotein on the surface of many cell types and serves as a primary receptor for human adenoviruses. The knob domain of the homotrimeric fiber binds to the coxsackie-adenovirus receptor, mediating virus attachment to the cell. Subsequently, an Arg-Gly-Asp-motif located on the penton base interacts with a cell surface integrin molecules that play a role as a secondary or internalization receptor and triggers the virus entry via clathrin-dependent receptor-mediated endocytosis. In addition to the coxsackie-adenovirus receptor, other receptors, such as class I major histocompatibility complex, heparan sulphate glycosaminoglycans and vascular cell adhesion molecule-1 have been shown to play a role in the viral cell entry. Adenoviruses transduce both dividing and quiescent cells and they provide an efficient, but transient transgene expression. They exist extrachromosomally within the host cell, although the viral DNA migrates into the cell nucleus. Because of their broad host and cell range, adenoviral vectors have been extensively used in experimental models, as well as in clinical protocols.

Adenoviral vectors have been used in suicide gene therapy, anti-angiogenic gene therapy, oncoviral therapy, gene therapy based on immune modulation, gene therapy targeting oncogenes or tumour suppressor genes and gene therapy targeting tumour invasion and apoptosis. In addition, approaches such as gene delivery of antisense or small interference RNAs (siRNA) targeted to growth factors in order to increase the sensitivity of glioma cells to chemotherapeutics or to inhibit migration and invasion of glioma cells have been developed. The most extensively studied and used adenoviral serotype in gene therapy is the serotype 5, causing only mild or asymptomatic infections (Sharma et al, 2009, Raty et al, 2008). In the first generation of adenoviral vectors, the E1 region encoding for early viral proteins is replaced by the transgene, making the vectors replication defective. By deleting the E1 region, a transgene capacity of 4.7-4.9 kb is achieved, which is then further increased to 8.3 kb by deleting the non-essential region E3. To circumvent the problem of the host cellular immune response against the viral proteins produced by other early viral genes, a second generation adenoviral vectors was described, where the E4 region is deleted. This second generation of adenoviral vectors was shown to be safer in terms of causing less apoptosis and inducing to a lesser extent an immune response in vivo. The third generation adenoviral vectors were constructed by deleting additional adenovirus genes, resulting in a so-called high-capacity, gutless or helper-dependent adenoviral vectors.
3. Glioblastoma multiforme

Brain tumours can be divided into primary and secondary brain tumours. Primary brain tumours derive from within the brain, whereas secondary brain tumours originate from organs such as lung, breast, colon, skin, kidney and thyroid, which have metastasized to the brain. Primary brain tumours are classified based on histological appearance, resemblance of the tumour cells to embryonic, foetal or differentiated mature cells, growth pattern, radiological features and surgical appearance. The most common and also the most lethal primary brain tumours in adults are gliomas, which account for 60 to 70% of primary tumours. Their overall global incidence lies between 4-6 new cases per 100,000 population per year, which has not changed significantly within the past 30 years (Ohgaki & Kleihues, 2005, Ohgaki, 2009)(Wirth et al, 2009). Current standard therapy of malignant glioma includes surgical resection followed by adjuvant radiotherapy and/or chemotherapy (i.e. temozolomide), which results in an overall mean survival of 14.6 months after diagnosis and a 5-year survival of 9.8% (Stupp et al, 2009). More recently, in May 2009 the FDA approved bevacizumab (an antibody against the growth factor VEGF) for the treatment of glioblastoma multiforme under an accelerated approval process (Cloughesy, 2010). The approval was based on the results of 2 phase II clinical trials that showed bevacizumab reduced the tumour size in some patients. Another recently published phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan showed a significant therapeutic activity in patients with recurrent glioblastoma (Friedman et al, 2009). However, the results are somewhat under strong dispute, as the European Medicine Agency (EMA) has recently denied the approval of bevacizumab for the treatment of recurrent high-grade glioma (Balana et al, 2011). Nevertheless, even though the addition of temozolomide to standard therapy, as well as the addition of bevacizumab plus irinotecan as an optional protocol, has significantly improved the survival of glioma patients, there is still a highly unmet medical need for this indication. Even recent trials combining cetuximab (an antibody against the epidermal growth factor receptor) with bevacizumab and irinotecan or the combination of bevacizumab with temozolomide did not improve the overall survival of patients with glioblastoma multiforme (Hasselbalch et al, 2010, Lai et al, 2011). It is the diffusely infiltrating property of these tumours and the resistance to chemotherapy and radiotherapy that makes the therapy mostly ineffective, which ultimately leads to tumour recurrence at some point of time.

3.1 Gene therapy of glioblastoma multiforme

Brain tumours bear several features that make them particularly amenable to gene therapy. Firstly, brain tumours are in most cases single, localized lesions of rapidly dividing cells in a background of non-dividing cells. Secondly, they rarely metastasize outside the central nervous system and if recurrence occurs, it typically happens in the close vicinity of the original lesion. Different gene therapy approaches using different gene transfer vectors for the treatment of malignant gliomas have been studied. These include pro-drug activation/suicide gene therapy, anti-angiogenic gene therapy, oncolytic virotherapy, immune modulation, correction of gene defects, inhibition of tumour invasion, apoptosis induction, gene therapy to enhance chemo- and radiotherapy, myeloprotective gene therapy, antisense and RNA interference (RNAi) based strategies. Although most of these methods have demonstrated promising success in vitro and in pre-clinical studies, only few of them have progressed up to phase III clinical trials.
3.2 Clinical efficacy of adenovirus mediated gene therapy in glioblastoma multiforme

Up to date, 20 registered clinical trials for the treatment of malignant glioma using adenoviral vectors have been conducted or are still ongoing. Different approaches such as suicide gene therapy strategy, interleukin-2, interferon-β, melanoma differentiation associated gene-7/interleukin-24 (mad-7/IL-24), p53, a dominant negative EGF receptor mutant (D-EGFR-Cd33), the human soluble FMS-like tyrosine kinase 3 ligand concomitant with Herpes simplex virus-thymidine kinase, a Fas-TNF receptor chimera, p53 and a conditionally replication-competent adenovirus (Delta-24-RGD) have been utilized. The very first clinical gene therapy trial against brain cancer was registered in 1992. In that trial autologous tumour cells were modified ex vivo with retrovirus to express interleukin-2 gene in neuroblastoma. In the following year, brain cancer patients were treated with herpes simplex virus thymidine kinase suicide gene therapy using retrovirus vectors producing cells and concomitant administration of ganciclovir. However, transduction efficiency was a major problem in these trials, resulting in poor therapeutic efficacy. Another problem was that retrovirus producing cells were murine of origin. In comparison to retroviral vectors, adenoviral vectors have shown to have higher transduction efficacy as well as transgene expression (Sandmair et al, 2000a). Adenoviruses transduce both, dividing and quiescent cells, which may provide an important advantage, as not all cancer cells proliferate within the tumour at a given time point. In 1996, Eck et al. published the first phase I clinical trial, where adenovirus Herpes simplex virus-thymidine kinase was used with the intention to patients with recurrent gliomas. However, the first completed trial using adenovirus Herpes simplex virus-thymidine kinase in patients with malignant glioma was published by Sandmair et al. in 2000. In that study, 21 patients were enrolled to compare the efficacy of both, the retrovirus-packaging cells Herpes simplex virus-thymidine kinase and the adenovirus mediated Herpes simplex virus-thymidine kinase gene therapy for the treatment of primary or recurrent gliomas. The mean survival time in the adenovirus Herpes simplex virus-thymidine kinase group was 15 months and significantly longer, when compared to a 7.4 months survival time in the retrovirus-packaging-cells group. The control group, which received adenovirus LacZ had a mean survival time of 8.3 months. Although the retrovirus-packaging-cells approaches were found safe, no efficacy was observed in malignant glioma patients. The low gene transfer efficacy with retrovirus and the lack of the treatment response indicated that retroviral Herpes simplex virus-thymidine kinase gene therapy may not be efficient enough in human clinical settings. This was further confirmed by the results from the first randomized, open-labeled, parallel group phase III clinical trial of 248 patients, where Herpes simplex virus-thymidine kinase produced by retroviral producing cells did not result in an improvement of survival (Rainov, 2000). Trask et al. conducted a non-controlled phase I study in which patients with recurrent malignant brain tumours were injected with Herpes simplex virus-thymidine kinase with doses ranging from $2 \times 10^9$ to $2 \times 10^{12}$ virus particles via a single stereotactic intratumoural injection (Trask et al, 2000). Despite the occurrence of pronounced central nervous system toxicity in patients receiving the highest dose, adequate safety and feasibility was reported for adenovirus Herpes simplex virus-thymidine kinase gene therapy.

In 2003, a phase I clinical trial published by Lang et al., described the use of adenoviral vectors encoding for the tumour suppressor gene TP53 to treat patients with recurrent malignant gliomas (Lang et al, 2003). In that study, 15 patients ought to undergo
intratumoural stereotactic injection of the adenoviral vector via an implanted catheter, followed by en bloc resection of the tumour and treatment of the post-resection cavity. Due to the design of the study, the tumour response could not be assessed, but the study demonstrated minimal toxicity. No systemic viral dissemination was observed and a maximum tolerated dose was not reached in this study. Analysis of tumour specimens demonstrated restricted transgene expression close to the injection site. Chiocca et al. published a phase I dose-escalation trial of the oncolytic adenovirus ONYX-015, which preferentially replicates in p53-deficient cells and thereby lyses them (a common feature in tumour cells). In that trial, 24 patients with recurrent malignant glioma were injected with the oncolytic virus with doses ranging from $10^7$ to $10^{10}$ pfu (plaque forming units) in a total of 10 injections into 10 different sites of the cavity of resected tumours. None of the patients experienced serious adverse events related to the virus. However, in this trial the maximum tolerated dose was not reached. All patients showed tumour progression with a median time of 46 days and a median survival time of 6.2 months (Chiocca et al, 2004). One patient with anaplastic astrocytoma had stable disease and two patients who underwent a second resection had lymphocytic and plasmacytoid cell infiltration at the site of injection. Nevertheless, despite a good safety profile, the overall therapeutic efficacy was poor. In another study performed by Chiocca et al., 11 patients were injected with different doses of interferon-β-expressing adenoviruses ranging from $2 \times 10^{10}$ to $2 \times 10^{11}$ viral particles stereotactically into the tumour. This was followed by surgical removal of the tumour 4-8 days later with additional injections of the adenovirus into the tumour bed (Chiocca et al, 2008). Generally, the treatment was well tolerated with only one patient experiencing dose-limiting side effect after post-operative injection with the highest dose. However, all patients had disease progression and/or recurrence within 4 months after the treatment. The median time to tumour progression was 9.3 weeks and the median overall survival was 17.9 weeks. So far, the only adenoviral vector that has completed a phase III clinical trial (Cerepro®, by Ark Therapeutics Group plc) is based on the suicide gene therapy Herpes simplex virus-thymidine kinase. In that trial, adenoviral vector encoding for Herpes simplex virus-thymidine kinase was injected into the walls of the tumour cavity of glioma patients after the resection of the tumour (Figure 3). The clinical efficacy of Cerepro® was evaluated first in two separate phase II clinical trials; a phase IIa trial and a phase IIb trial (Sandmaier et al, 2000a, Immonen et al, 2004). In the randomized and controlled phase IIb trial published by Immonen, carried out in 36 patients, seventeen patients with operable or recurrent malignant gliomas receiving Herpes simplex virus-thymidine kinase adenoviral vector (Cerepro®) implicated a survival advantage over control patients, who did not receive Cerepro®. The mean survival of the patients in the Cerepro® group (70.6 weeks) was significantly longer ($p<0.0095$) when compared to the standard care group (39.0 weeks) or a historical control group ($P<0.0017$). This study was also historically the first randomized, controlled trial with an adenoviral vector using Herpes simplex virus-thymidine kinase, where an increased survival of the patients was shown when compared to standard therapy. The results from the study were very encouraging and it was concluded that Cerepro® could provide an effective adjuvant treatment for patients with operable primary or recurrent malignant glioma. Therefore, a multicenter, standard care controlled, randomized clinical phase III trial was commenced. However, the results coming out of that trial were not as significant as those from the
previous IIb trial. As a result, suggestions by the European Medicines Agency were given for further clinical evaluation, as they concluded that the data did not provide sufficient evidence of significant clinical benefit compared to current standard treatment.

Fig. 3. In case of Cerepro® adenoviral vectors encoding for the Herpes simplex virus-thymidine kinase are injected into the tumour cavity of resected tumours, which is followed by ganciclovir administration. The injection of Cerepro® into the tumour cavity results in the transduction of mainly healthy brain cells, which express the Herpes simplex virus thymidine kinase and converts ganciclovir into ganciclovir-monophosphate. Ganciclovir-monophosphate is then further converted ultimately into ganciclovir-triphosphate, by the cells own kinases, which is toxic to proliferating cells. As the brain does not harbour any proliferating cells other than the tumour cells, Cerepro® does not affect healthy brain cells.

4. Suicide gene therapy

The most common approach in these studies has been the use of the Herpes simplex virus-thymidine kinase gene followed by either ganciclovir or valacyclovir treatment. Suicide gene therapies can generally be divided into two steps. In the first step, a gene of a foreign enzyme is delivered (via viral vectors) to the tumour where it is to be expressed. In the second step, a non-active pro-drug is administered, which will be selectively metabolized to the active form by the foreign enzyme expressed in the tumour. Ideally, the gene for the enzyme should be expressed exclusively in the tumour cells and should reach a concentration sufficient to activate the pro-drug for a clinical benefit. Several suicide genes have been studied with varying results. Herpes simplex virus-thymidine kinase, Cytosine deaminase (CD)/5-fluorocytosine (5-FC), cytochrome P450/cyclophosphamide (CPA), E.coli purine nucleoside phosphorylase (PNP)/6-methyl-purine-2'-deoxynucleoside, and carboxypeptidase G2 (CPG2)/methotrexate-a-phenylalanine are some of the pro-drug activation systems that have been attempted on brain cancer treatment. Because the foreign
enzyme will not be expressed in all cells of the targeted tumour in vivo, a "bystander effect" is required, whereby the pro-drug is cleaved to an active drug that kills not only the tumour cells in which it is formed, but also neighbouring tumour cells that do not express the transgene (Figure 4).

Fig. 4. A schematic presentation of how suicide gene therapy works. First, a suicide gene needs to be introduced into the target cells, after which it will be expressed by the cell. The second step is the administration of a pro-drug, which will be converted into a toxic metabolite by the introduced pro-drug activating enzyme (i.e. the suicide gene), which ultimately leads to cell death. The toxic metabolite can further diffuse passively or actively into neighbouring tumour cells and induce cell death.

4.1 Herpes simplex virus-thymidine kinase / ganciclovir therapy against glioblastoma multiforme
The Herpes simplex virus-thymidine kinase/ganciclovir therapy is based on the pro-drug activating enzyme Herpes simplex virus-thymidine kinase that converts the nucleotide analogue ganciclovir to its toxic metabolite. Apart from malignant glioma, Herpes simplex virus-thymidine kinase/ganciclovir has been studied also in many other cancer types, both in pre-clinical models and in clinical trials. Data from six phase I/II clinical trials using adenoviral vectors encoding for the Herpes simplex virus-thymidine kinase have been published and two of the trials have demonstrated a significant benefit for the treatment of patients with malignant glioma. Despite not observing total cures or complete responses, the
prolongation of survival without a deteriorating quality of life in patients with a fatal disease such as glioblastoma multiforme is a strong indication of the therapeutic potential of gene therapy.

Originally, the Herpes simplex virus-thymidine kinase gene was cloned by McKnight in 1980 (McKnight, 1980). This property to kill tumour cells when given ganciclovir was soon realized and led to the idea of using this as a therapeutic strategy to treat solid tumours. The first proof-of-concept of the therapeutic efficacy of the Herpes simplex virus-thymidine kinase/ganciclovir therapy was established ten years later by Moolten and Wells. They used retroviral vectors expressing the Herpes simplex virus-thymidine kinase gene in order to transduce murine sarcoma and lymphoma cells in vivo (Moolten & Wells, 1990). In the initial studies murine fibroblasts transduced with Herpes simplex virus-thymidine kinase retroviral vectors were inoculated intratumourally, followed by ganciclovir treatment (Moolten & Wells, 1990). Ganciclovir is a synthetic acyclic analogue of 2'-deoxy-guanosine, chemically designated as 9-[[2-hydroxy-1-hydroxymethyl)ethoxy]methyl]-guanine. Ganciclovir is known as being the first antiviral drug to be effective in the treatment of cytomegalovirus (CMV) diseases in humans. It is converted to ganciclovir-monophosphate by the Herpes simplex virus-thymidine kinase, which has a 1000 fold higher affinity for ganciclovir than the mammalian form of the enzyme. Cell kinases continue the phosphorylation further and convert the ganciclovir-monophosphate into a ganciclovir-diphosphate and finally to a toxic ganciclovir-triphosphate. Cytotoxic ganciclovir-triphosphate results in the inhibition of the DNA polymerase thus preventing DNA replication (Figure 5).

The Herpes simplex virus-thymidine kinase/ganciclovir therapy is cell cycle dependent, where only dividing cells are affected. This property is considered to be of advantage in cancer therapy, where dividing tumour cells are often surrounded by non-dividing, healthy cells. Moreover, it has been shown that the expression of the non-human enzyme Herpes simplex virus-thymidine kinase and the presence of tumour antigens that become available after the death of the transduced cells are able to induce an antitumoural immune response against the tumour cells. It is believed that both local and systemic immune responses contribute to the overall therapeutic effect of Herpes simplex virus-thymidine kinase/ganciclovir by stimulating the tumour infiltration of CD4+ and CD8+ T cells, natural killer cells and macrophages into the tumour. Furthermore, work by different groups suggests that ganciclovir induced cell killing occurs due to apoptosis through the activation of mitochondrial damage pathways, chromosomal aberrations, and sister chromatin exchange that leads to target cell death due to cell cycle arrest.

As current gene therapy vectors cannot achieve 100% gene transfer efficacy, the bystander effect is essential for an effective anti-tumour therapy. A very important advantage of Herpes simplex virus-thymidine kinase/ganciclovir gene therapy is its bystander effect, where neighbouring non-transduced cells are also exposed to the toxic ganciclovir-triphosphate metabolite (Freeman et al, 1993). Interestingly, it was noticed that also Herpes simplex virus-thymidine kinase-negative cells were killed after exposure to ganciclovir. Moreover, it was found that even with low concentration of Herpes simplex virus-thymidine kinase-positive tumour cells, tumour growth could be prevented (Freeman et al., 1993, Wu et al., 1994, Sandmair et al., 2000). An experiment conducted by Sandmair et al suggests that 10% of the tumour cells have to be transduced with Herpes simplex virus-thymidine kinase gene in order to achieve a significant tumour regression (Sandmair et al, 2000). However, this “bystander effect” seems to be dependent on the cell type. The exact
The mechanism of the “bystander effect” is not completely understood, but several mechanisms have been postulated (van Dillen et al, 2002). For example, it has been observed that the number of gap junctions plays a vital role in this effect. Additionally, it was demonstrated that the number of gap junctions seems to correlate with the efficiency of killing neighbouring cells. There is on the other hand experimental evidence where the release of toxic ganciclovir-triphosphate into the culture medium by dying cells results in apoptosis of non-transduced cells. This was shown to be dependent on ganciclovir-triphosphate accumulation in the surrounding cells and the concentration of ganciclovir in the medium, suggesting that cell-to cell contacts are not essential. Another possible mechanism includes the stimulation of the immune system, endothelial cell transduction leading to disruption of tumour vasculature and phagocytosis of apoptotic vesicles by neighbouring non-transduced cells (van Dillen et al, 2002, Burrows et al, 2002, Kruse et al, 2000).

Fig. 5. In Herpes simplex virus-thymidine kinase/ganciclovir gene therapy the gene for the Herpes simplex virus thymidin kinase is introduced into the target cells. After gene transfer the pro-drug ganciclovir is administered to the patient, which is converted by the introduced Herpes simplex virus thymidin kinase into ganciclovir monophosphate. The cells own enzymes than further convert ganciclovir-monophosphate into ganciclovir-biposphate and ganciclovir-triphosphate, being the active molecule. Ganciclovir triphosphate is a nucleotide analogue, which is incorporate into the DNA genome, thereby halting DNA replication.
5. The safety of adenoviral vectors

Adenoviruses are human pathogens and a significant proportion of the human population posses pre-existing antibodies against them. Some serological surveys have shown this to be as high as 40-60% for serotypes 1, 2 and 5 in children, which could rapidly inactivate the systemically administered adenoviral vectors (Verma & Weitzman, 2005). Generally, an adenoviral vector injection will result in an initial non-specific host response with the release of the cytokines tumour necrosis factor-α (TNF-α), interleukins (IL) 1 and 6, followed by a specific cell-mediated immune response directed against the infected cells, mediated by cytotoxic T lymphocytes, monocytes and natural killer cells and a humoral response through activated B cells and CD4+ T lymphocytes. This may give rise to a major obstacle for the efficiency and safety of the use of adenovirus vectors (Barcia et al, 2006, Driesse et al, 1998).

The immune response within the brain towards a viral vector, however, might differ from the response one can see in other organs. Immunologically, the brain can be divided into two compartments and the type of the initiated immune response as a result of viral vector delivery (into the brain) will greatly depend on the compartment into which the viral vector has been delivered. The first immunological compartment consists of the ventricles, meninges and choroid plexi, which all contain cellular, vascular and lymphatic components of the immune system as one can see in most other organs (Galea et al, 2007). The second immunological compartment is the brain parenchyma itself. A hallmark of this compartment is the lack of dendritic cells and lymphatic vessels in it and it is separated from the general circulation by the blood brain barrier, formed by tight intercellular endothelial junctions (Galea et al, 2007, Bechmann et al, 2007). Functionally, this means that the injection of viral vectors into the ventricular systems, for example, will result in the activation of an innate and adaptive immune response, whereas the injection of a viral vector only into the brain parenchyma would result in an innate inflammatory response, but would not trigger a systemic adaptive immune response (Lowenstein et al, 2007). Furthermore, Thomas et al demonstrated in his studies that the innate immune response was dose-dependent and ultimately influenced the duration of the transgene expression (Thomas et al, 2001).

Despite this, the safety data of adenoviral mediated gene therapy collected from different human trials have been uniformly satisfactory. Although, there is not much data about the long-term aspects regarding the safety of adenoviral vectors in humans, several meta-analysis exist demonstrating that adenoviruses have an adequate safety profile in humans. The tolerability towards adenoviral vectors has been acceptable and the side effects have mostly been mild without any serious adverse events related to gene therapy (Wirth et al, 2009, Immonen et al, 2004). Generally, the main concerns in cancer gene therapy of patients with malignant gliomas are the safety issues when using viruses to deliver the therapeutic genes into the brain. Especially in the case of adenoviruses the inflammatory reactions that might arise in the brain are of concern. In some clinical studies using adenoviruses for gene transfer, evidences of side effects such as increased brain oedema, epileptic seizures and haemorrhages have been demonstrated (Wirth et al, 2009, Immonen et al, 2004). However, these complications have not been related directly to viral mediated gene therapy, but most likely to the advanced disease and the surgical procedures.

The first study evaluating the safety and efficacy of Cerepro® was also the first completed recombinant adenovirus mediated Herpes simplex virus-thymidine kinase gene therapy.
trial against malignant glioma in humans (Sandmair et al, 2000a). In order to evaluate the safety of Herpes simplex virus-thymidine kinase therapy serological assessment, i.e. routine blood and urine analysis, as well as tests for the detection of anti-adenoviral antibodies were performed. The study showed that the therapy was well tolerated and no major alterations in routine laboratory tests were observed. No systemic escape of the virus was detected from urine or plasma samples with PCR. Anti-adenovirus antibodies were measured before and two weeks after the gene transfer. A four fold increase in antibodies against the adenoviral vector was detected in four patients. Two of the patients had also short-term transient fever reactions. No severe adverse events related to viral mediated gene therapy were detected in neither of the treatment groups. The most serious adverse events were some elevation in the frequency of epileptic seizures (2 patients), hemiparesis and aphasia (1 patient). Nevertheless, it could not be excluded that these adverse events were related to the mechanical irritation of the operation procedure and the tumour resection itself.

The phase IIb study of Cerepro® showed also a good safety profile with no signs of significant safety concerns. Three patients had transient reversible elevation of liver enzymes; two out of 17 patients in the Cerepro® group developed localized post-operative intracerebral oedema. Adenovirus was detected by PCR in the plasma of two patients three days after gene therapy, but not thereafter. A similar safety profile was seen also in the phase III trial.

6. Ethical considerations of gene therapy based medicines

As a result of the study of Cline and colleagues in 1980, an intense debate about the ethical issues of gene therapy was initiated. It is of no surprise that genetic modification of human beings, even though for therapeutic purposes, would raise many ethical questions. Arguable, gene therapy raises many ethical questions, which is a response to uncertainty and fear towards gene therapy or its possible consequences. It raises concerns about the safety in humans and their offspring, environment safety, its impact on the society in general and whether gene therapy is ethically distinctive from other medical therapies. An example of the social impact of gene therapy based medicine would be, whether it is going to be a treatment modality only accessible to a certain group of people, i.e. people with a higher social status or income. Is it going to be accessible for everyone, in other words will it be covered by the social health care system? How much it will cost? It is of no surprise that opinions and points of views about gene therapy vary from one extreme to another. Cultural as well as religious opinions have a strong impact on these standpoints. Questions, such as which would be the diseases where gene therapy is ethically acceptable or what would be the cost of the therapy, need to be asked in order to justify gene therapy in humans.

Whereas gene therapy is more tolerated for life-threatening diseases, such as cancer or AIDS, it is not tolerated in the correction of learning disorders. The question is what is acceptable and what is not? How about dealing with genetic or chromosomal disorders? Would it be ethically acceptable to practise gene therapy on people with Dawn syndrome? What would be the justification of using gene therapy in the enhancement of some individual physical or mental properties? Somatic gene therapy appears to be more tolerated than germ-line gene therapy. Currently, legislation allows only gene therapy into somatic cells, even though the distinction between germ-line gene therapy and somatic gene therapy can be questioned. One of the main arguments and at the same time also one of our biggest fears, is the risk of uncontrolled genetic changes produced in an individual by gene
therapy, which in the worst case would be passed also onto the offspring’s of the treated person. The fact, that other therapies also can cause genetic alterations is often disregarded. For example, many different mutagenic drugs (e.g. those often used in cancer treatment), as well as radiation therapy may cause genetic alterations and if this mutation happens in germline, it will be passed onto future generations.

There are also technical issues concerning the justification of gene therapy. For example, what are the technical details of the DNA and vector to be used? The technical aspects involved, risks endeavoured by the patient and the fear of human genetic engineering are some of the major reasons why human gene therapy trials have long been difficult to conduct. The use of viral gene transfer vectors, such as lentiviruses has raised scepticism about the safety of these vectors. Non-viral vectors are not yet efficient enough, but have gained better acceptance in the society. In this regard, it seems that gene therapy in cancer is ethically acceptable, whereas the use of genetically modified stem cells is a much more difficult topic. Needless to say, the normal principles of good clinical research apply in the conduct of the ethical evaluation of gene therapy protocols. The integrity and free will of a patient should be respected, all available information for the informed consent should be given and the safety of an individual must be the first concern of the treatment protocols.

7. Conclusion

Currently, most of the gene therapy strategies used are limited to the local administration of the gene transfer vector, or to \textit{ex vivo} gene transfer approaches. For that, malignant glioma represents an attractive target for gene therapy, because of its restricted anatomical location and absence of metastases outside the central nervous system. However, the greatest shortcoming with gene therapy is the low transduction efficiency of the gene transfer vector and its minimal distribution within the tissue. Certainly, the low transduction efficiency can be regarded as a methodological problem to some extent, but still, if one wants to improve the potential scope of gene therapy, the focus needs to be directed towards vector development and improved efficiencies. Furthermore, the concept of using a single agent therapy has been noted as being not as successful as being hoped in achieving a complete cure, and thus, combination therapy with existing conventional modalities or other new therapies may offer additional benefit in cancer gene therapy. There are already encouraging results of combination gene therapy in experimental glioma, where adenoviral mediated Herpes simplex virus-thymidine kinase/ganciclovir therapy has been combined to adenovirally transduced FMS like tyrosine kinase 3 ligand (FLT3L) immunotherapy (King et al, 2008).

Currently, the first gene based products have entered the market. In October 2003, China became the first country to approve the commercial production of a gene therapy drug. Shenzhen SiBiono GenTech (Shenzhen, China), obtained a drug license from the State Food and Drug Administration of China (SFDA; Beijing, China) for its recombinant Ad-p53 gene therapy (Gendicine) for head and neck squamous cell carcinoma (HNSCC). In 2006, the conditionally replicating adenovirus H-101 gained marketing approval for head and neck squamous cell carcinoma - also in China. More recently, Rexin-G, a pathotropic targeted retroviral vector designed to interfere with cyclin \textit{Gi} gene by integrating into the host DNA,
has recently been approved in the Philippines for the treatment of all solid tumors that are refractory to standard chemotherapy.

8. References


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