Radiation Therapy and Gene Therapy: a Potential New Combined Modality in the Management of Malignant Diseases

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Abstract

Despite of recent advances in the surgical management, chemo- and radiation therapy of malignant diseases, cancer is one of the leading causes of mortality. Therefore, development and clinical application of new therapeutic modalities, such as gene therapy is necessary. The new therapeutic approaches should be applied together with the existing treatment protocols. Radiation therapy is an excellent candidate for combining with gene therapy. Here we summarize the recent data which provide evidence that various forms of cancer gene therapy, such as the activation of the anti-tumour immune response, gene directed enzyme pro-drug therapies and oncolytic viruses might be very efficiently combined with local tumour irradiation. Gene therapy might improve the radiosensitizing effect of many drugs and radiation can drive the tumour-specific expression of therapeutic genes, as well. Even tumour hypoxia that causes radiation resistance might be utilized to improve the combined effects of radiation- and gene therapies. So far, three gene therapy protocols have been accepted for phase 4 clinical trials to treat cancer patients.

Key Words: cancer, radiation, gene therapy, hypoxia, radiosensitizers

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Received: 11th December 2017
Accepted: 14th December 2017

INTRODUCTION

Gene therapy is a promising new therapeutic modality in the field of cancer treatment. There are many gene therapy approaches that might be beneficial when treating cancer patients. The potential modalities include the activation of the immune system against the tumour, the application of the gene directed enzyme pro-drug therapy (GDEPT) and oncolytic viruses. Some of these approaches have already been tried in the clinical practice and other clinical trials are undergoing. The various cancer trials include the treatment of brain tumours, melanomas, lung, liver and renal cancer and other tumours, as well. Despite of the success of numerous animal experiments, the clinical applications have not presented many promising data, so far. One of the possible explanations for the unfavourable results is that mainly cancer patients in the final stage of their progressive diseases were included in the trials. Other possibility is that using the first generational vectors, only a small portion of the tumour cells will contain the therapeutic genes, and despite of the potential bystander effect, that is not sufficient for tumour cure. Nonetheless, these trials proved the safety of the various gene therapy protocols and might serve as a basis for the establishment of more efficient new therapeutic combinations.

The new anti-cancer modalities should be used in conjunctions with existing modalities. Radiation therapy might be an excellent candidate for combining with gene therapy. In this paper we summarize how the various gene therapy protocols might be combined with tumour irradiation.

RESULTS

Activation of anti-tumour immune attacks: combination with radiation therapy

There are several immune-therapy approaches that could increase the immunogenicity of the tumours (Shawler et al., 1997). One possibility is the introduction of cytokine encoding genes into the tumour cells. This can be achieved either by direct intra-tumour injection of viral vectors or by *ex vivo* modification of the malignant cells. The direct intra-tumour vector injection is much simpler than the *ex vivo* modification, but there are certain risks arising from the introduction of large number of viral particles into the human body. During the *ex vivo* approach most of the tumour is removed by surgery and first a primary cell culture is established from the malignant tissue. Then cytokine encoding vectors are introduced into the *in vitro* growing tumour cells and cell division is stopped by high dose irradiation of the culture. Finally, the cytokine expressing irradiated tumour cells are used to vaccinate the same patient from whom the original tumour was removed. It is expected that the host immune system is activated by the vaccine, and it will attack both the residual tumour cells at the site of the surgery and the cells at distant metastases. The key requirement of this protocol is the presentation of tumour-associated antigens in the microenvironment of cytokine secretion (Dranoff et al., 1993; Allione et al., 1994)

Li et al. (1998) and Staba et al. (1998) reported that the combination of radiation with intra-tumour administration of a cytokine (TNF-alpha) encoding vector substantially slowed down tumour progression. Lumniczky et al. (2002) found that cytokine expressing vaccines might cure about 30-40% of brain tumour bearing mice. Local radiation therapy alone hardly increased

life span; however, the combination of these two modalities improved survival rates up to 80-100%. One simple explanation for the synergistic effect of vaccination and radiation therapies is that there is a continuous competition between tumour growth and tumour eradication by the activated immune system. Local irradiation decreases the tumour burden, so the activated immune system could overcome the decreased tumour mass. Another possibility is that after irradiation the primary tumour cells die by necrosis. The necrotic death might lead to the liberation of immunogenic molecules that further enhances immune response.

Gene directed enzyme pro-drug therapy: effect of radiotherapy and conventional radiosensitizers

During cancer chemotherapy a principle problem is the frequently observed acquired resistance to the drugs. Gene directed enzyme pro-drug therapy (GDEPT) with drug-sensitizing genes is a promising new tool to overcome resistance and decrease the unfavourable side effects of chemotherapy. In GDEPT tumour cells are transduced with so-called suicide genes. The enzyme product of the suicide genes can convert potentially non- or mildly toxic drugs to highly toxic agents. After systemic drug administration the suicide gene-modified tumour cells will metabolize the anti-tumour agent, so higher concentration of active metabolites is achieved within the tumour mass that leads to selective, increased cell killing.

The most frequently used GDEPT protocol is the thymidine kinase/ganciclovir system. The *Herpes simplex* derived thymidine kinase (TK) converts ganciclovir (GC) to ganciclovir-monophosphate, which is further phosphorylated by cellular kinases to toxic ganciclovir-triphosphate. Ganciclovir-triphosphate incorporates into the DNA and inhibits DNA synthesis (Aghi at al., 2000; Takamiya et al., 1992; Maron et al., 1996). Mammalian cells lack TK, thus GC causes toxic effects only after transfecting cells with TK.

One of the most widely applied cancer chemotherapy agents is 5-flurouracil (5-FU). In mammalian cells 5-FU is metabolized first into nucleoside fluorouridine by uridine phosphorylase and then phosphorylated into 5-fluoro-2'-uridine-5'-monophosphate (FUMP) by uridine kinase. FUMP incorporates into RNA through FUTP or further metabolized into FdUMP. FdUMP is a potent inhibitor of thymidylate synthase, a key enzyme in the synthesis of dTMP, which is a precursor of DNA replication (Kanai et al,. 1998). Unfortunately, 5-FU resistance and toxic side effects are frequent in cancer patients. There might be two possibilities to overcome this problem. One of them is to produce 5-FU from the non-toxic 5-fluorocytosine (5-FC) by bacterial or yeast cytosine deaminase enzymes (CD) through GDEPT (Aghi at al., 1998; 2000). Another possibility is to introduce the E. coli uracil phosphoribosyl-transferase (UPRT) gene into the tumour cells. UPRT converts 5-FU directly and very efficiently into FUMP (Inaba et al,. 1999).

Desaknai et al. (2003) used a double-suicide GDEPT system against murine brain tumours. The applied adenoviral vector encoded both the TK and the UPRT genes. Intra-tumour injection of this vector and subsequent treatment with the corresponding agents substantially slowed down tumour progression. They have found that this protocol might be very efficiently combined with irradiation. Under *in vitro* circumstances the combination of 5-FU and ganciclovir treatments with irradiation increased cytotoxicity by three orders of magnitude. In glioma-bearing mice the combined treatment also improved survival compared to a single agent modality. This was in accordance with other findings that the 5-FC/CD system had a radiosensitizing effect in

animal models of head and neck (Hamstra et al., 1999), colorectal (Kievit et al., 2000, Gabel et al., 1998) and 9L glioma tumours (Kim et al., 1998).

Radiosensitizers are substances, which sensitize cells to radiation via different mechanisms. Systemic administration of different radiosensitizers, followed by irradiation of the tumour is well documented. However, by this way the radiosensitizer effect is non-specific and largely depends on the active drug concentration at the tumour site. There are possibilities to increase the sensitivity of the tumour cells towards the effect of radiosensitizers by the GDEPT approach. If a radiosensitizer is converted to the toxic form by cellular enzymes, then the introduction of the metabolic enzyme into tumour cells might increase the sensitizing effect. For this purpose one very promising candidate is Gemcitabin which has a known radiosensitizing effect. A recent study showed a strong correlation between the deoxycytidine kinase (dCK) activity of cells and Gemcitabin toxicity (Gregoire et al., 2002). Beside sensitizing the cells to Gemcitabin, increased dCK levels enhance the cytotoxic effect of aza-cytidine, as well (Hapke et al., 1996; Manome et al., 1996).

In our own experiments deoxycytidine kinase overexpression substantially increased both the toxic and radiosensitizing effect of Gemcitabine in human, mouse and rat glioma cell lines, but the enhancement rate of the treatment combinations varied: it was mild in the mouse Gl261 cells and much stronger in the rat C6 and human U373 cells. *In vivo* experiments showed a mild radiosensitizing effect of dCK overexpression both in the Gl261 and C6 animal models. The combination of dCK overexpression, Gemcitabine treatment and irradiation led to significant improvement in the survival rate of C6 bearing rats (66% survival) (Szatmári et al., 2008).

So far, two of the GDEPT protocols reached phase 3 clinical trial stage. Rainov et al. (2000) reported a multinational phase 3 trial where 248 patients with newly diagnosed, previously untreated glioblastoma multiforme were equally divided into a control and a gene therapy group. The control patients were treated by surgery, followed by radiation therapy. In addition to the standard therapy the gene therapy group were locally injected during surgery with a packaging cell line producing a Moloney murine leukemia virus derived retroviral vector that encoded the *Herpes simplex* thymidine kinase gene and then treated by ganciclovir. Unfortunately, the therapeutic outcome of the gene therapy group did not significantly differed from the outcome of the control patients. In another phase 3 trial Westphal et al. used a first generation replication-deficient adenovirus containing the herpes-simplex virus thymidine kinase gene in combination with intravenous ganciclovir administration and standard therapy. Patients receiving the gene therapy treatment had improved time to death, but did not show improvement in overall survival (Westphal et al., 2013).

GDEPT and hypoxic radiosensitizers

One of the major problems in radiation therapy is the radiation resistance of hypoxic cells. Hypoxic radioresistance might be overcome by the addition of hypoxic radiosensitizers. The first generational hypoxic radiosensitizers have not proved very efficient in clinical trials because of their strong systemic toxicity. In the range of the new generational hypoxic radiosensitizers, tirapazamine is a bioreductive agent that has a 50-200-fold differential toxicity towards hypoxic cells. It is reduced by one-electron addition to a highly reactive radical that is able to react with DNA to abstract hydrogen, leading to strand breaks. There are several cellular enzymes, such as cytochrome P450 reductase, nitric oxide synthase and xanthine oxidase that

are able to reduce tirapazamine to its reactive form (Garner et al., 1999; Rauth et al., 1998). Paterson et al. (2002) combined bioreductive chemotherapy with hypoxia-directed gene therapy in the treatment of human fibrosarcoma xenografts and by this way they could significantly reduce the radiation dose necessary for 50% survival.

Restoration of wild type p53 status

It is well-known that the p53 tumour suppressor gene is mutated in a high percentage of human cancer. The p53 protein has basic roles in cell cycle regulation and in radiation response. The restoration of wild type p53 activity in tumour cells should have a high impact on tumour treatment. In accordance, the enhanced radiosensitivity of glioma cells was detected after transduction with wild type p53 encoding vectors (Badie et al., 1999; Broaddus et al., 1999; Gridley et al., 1998). The radiosensitizing effect of p53 is probably established through its pro-apoptotic effect, but p53 might also suppress tumour vascularization.

A replication-impaired adenoviral vector that carries the p53 gene (Advexin), had been evaluated in phase 2 and 3 clinical trials to treat head and neck cancer patients. Patients having tumours with damaged p53 gene experienced significantly improved 6-12 months survival compared to those treated with methotrexate (Nemunaitis and Nemunaitis, 2011). Despite of the early positive results clinical trials with Advexin were halted. Quite interestingly, clinical trials with a first generational adenovirus vector encoding the wild type p53 protein (Gendicine) were more successful in China. Gendicine was the first clinically approved gene therapy and tumour virotherapy drug in the world (Peng, 2005). The clinical trials with Gendicine showed that complete remission and overall response were significantly better in the Gendicine therapy group than in the group receiving only conventional treatments (Yuan et al., 2016).

Replication competent and oncolytic viruses

The first generational viral vectors were designed to not replicate in transduced cells. After intratumour delivery of these vectors, the viral infection is limited to cells surrounding the needle track. This low infection rate probably highly contributed to the very limited success of the former clinical trials. This problem might be overcome by the introduction of new generational viral vectors that are capable of propagation in tumour cells.

Some of the new replicative vectors might have oncolytic effects, as well. One of the first potentially replicative, oncolytic vectors was the ONYX adenovirus vector (Hann and Balmain, 2003). In the ONYX virus only the E1B region was removed from the wild type adenovirus. The E1B protein can bind to and inactivates the p53 tumour suppressor protein. The inactivation of the p53 protein will allow adenovirus replication in the infected cells. In the absence of the E1B region p53 should inhibit viral replication in normal cells. Because p53 is mutated in most of the cancer cells, the virus might replicate in and kill the p53 deficient tumour cells. The anticancer effect of the ONYX virus have been evaluated in several clinical trials including head and neck (Khuri et al., 2000) and metastatic lung tumours (Nemunaitis et al., 2001). ONYX is much more effective when combined with radiation in colon carcinoma (Rogulski et al., 2000) and glioma (Geoerger et al., 2003) tumour models. Although the clinical trials with ONYX were promising the application of this virus was halted in Western countries. Quite interestingly, however a very similar construct, called H101 gained approval from China's State Food and Drug Administration (SFDA) in 2005 for the treatment of head and neck cancer (Lu et

al., 2004; Garber, 2006). In H101 the E1B region and also part of the E3 region of the wild type adenovirus is deleted allowing the deficient virus to replicate only in p53 deficient cells. H101 is commercialized by a small biotech company called Sunway under the name Oncorine. In its phase III trial, Sunway reported a 79% response rate for H101 plus chemotherapy, compared with 40% for chemotherapy alone. Part of H101's success may be due to not treating manageable patient fevers, higher body temperature should aid viral replication and enhance the anticancer immune response.

Some viruses, such as vaccinia, measles, herpes simplex, Newcastle disease virus might preferentially replicate in tumour cells and demonstrate oncolytic (Lin and Nemunaitis, 2004) activities. Ionizing radiation can augment the oncolytic effect of herpes simplex (Stanziale et al., 2002), vaccinia (Timiryasova et al., 2003) and Newcastle disease (Safrany et al. unpublished data) viruses.

Combination of the oncolytic and immune system activating therapies

A special virus named OncoVEX$^{GM\text{-}CSF}$ (T-VEC) has been recently developed utilizing the oncolytic effect of the *Herpes simplex* virus and the immune activating effect of GM-CSF. In OncoVEX$^{GM\text{-}CSF}$ the gene modified *Herpes simplex virus* carries a gene encoding the human GM-CSF. This virus was intratumourally injected into melanoma, head and neck, colon/liver and pancreas tumour patients in various Phase I-III clinical trials (Kaufman and Bines, 2010). The most promising results have been detected in melanoma patients (Senzer et al., 2009; Andtbacka et al., 2015; Kaufman et al., 2016). In a phase III trial (Andtbacka et al., 2015) 436 unresected stage IIIB to IV melanoma patients were randomly assigned to a control and the gene therapy treated group. Median OS was 23.3 months with T-VEC and 18.9 months with GM-CSF treated controls. T-VEC efficacy was strongest in patients with stage IIIB, IIIC, or IVM1a disease. In October 2015, the US FDA approved T-VEC under the brand name Imlygic (https://www.imlygic.com/), for the treatment of melanoma in patients with inoperable tumours (US FDA, 2015) In January 2016 it was also approved in Europe for the treatment of inoperable melanomas.

Tumour vasculature

Tumour vasculature has high impact both on tumour growth and on the radiation sensitivity of tumours. Hypoxic regions might render the cells radio resistant, and on the other hand necrotic regions could substantially reduce tumour burden. The intra-tumour administration of a secretable angiostatin-like molecule into glioma xenografts by adenoviral injection had only marginal influence on tumour growth alone. However, its combination with radiation therapy resulted in a synergistic effect probably through the inhibition of tumour vascularization (Griscelli et al., 2000).

It was also shown that adenoviral transfer of the human iNOS gene enhanced the radiation response of human colorectal cancer. The radiosensitizing effect of iNOS occurred through the alterations in tumour vascularity and by inducing apoptosis in tumour cells (Wang et al., 2004).

Radiation driven gene expression

In cancer gene therapy it would be highly preferable if the therapeutic genes were expressed and/or the vectors replicated only in the targeted tumour cells. To achieve this, gene expression and/

or vector replication should be placed under the control of tumour specific promoters (Robson and Hirst, 2003). When cancer gene therapy is combined with radiation therapies there might be two possibilities to achieve tumour specific expression: the application of radiation induced promoters and the introduction of hypoxia induced promoters into the vectors.

Exposure of cells to ionizing radiation will activate a number of genes including, early and late response genes. The early response genes include c-jun, c-fos, EGR1, NFκB and $p21^{WAF1}$ (Robson and Hirst, 2003; Chastel et al., 2004). The EGR1 promoter is well characterized. It contains four copies of a $CC(AT)_6GG$ sequence, the so called CArG element that is responsible for radiation induction. Gene expression from the EGR1 promoter will be induced about 3-fold by 2 Gy irradiation (Manome et al., 1998). Synthetic promoters containing several CArG elements and a basal promoter might be created and linked to therapeutic genes. An adenoviral vector was constructed where the expression of TNFα was placed under the control of four CArG elements. Using this vector and tumour irradiation, effective concentrations of TNFα could be achieved locally in the tumours without systemic toxic side effects in clinical trials. When breast cancer, lung, rectum, pancreas tumour and melanoma patients were treated with the vector and tumour irradiation, very promising results were obtained (Weichselbaum et al., 2002).

The CArG element might be very efficiently used to drive gene expression from a TK construct after radiation (Joki et al., 1995), as well.

The $p21^{WAF1}$ promoter is also induced by radiation (Robson and Hirst, 2003) and it is sensitive to hypoxia. The iNOS gene was placed under the control of the WAF1 promoter and used in a murine fibrosarcoma model in combination with tumour irradiation. Significant tumour growth delay and apoptosis induction in the tumour were observed (Worthington et al., 2002). It was also proved that iNOS gene therapy in combination with the inducible WAF1 promoter resulted in a significant tumour cell radiosensitization (Worthington et al., 2004).

Hypoxia-induced gene expression

It is well known that severe hypoxia might be present in various human tumours. Tumour hypoxia is usually associated with aggressive disease and poor prognosis. However, tumour hypoxia might be utilized in cancer gene therapy by putting the therapeutic genes under the control of hypoxia responsible elements (HREs). HREs are enhancers containing the (A/G) CGT(G/C)(G/C) sequence and are present in the promoter region of several hypoxia responsive genes, such as vascular endothelial growth factor (VEGF), erythropoietin and phosphoglycerate kinase (Robson and Hirst, 2003). Hypoxia sensitive promoters are regulated through the binding of HIF1 transcription factor to HREs. HIF1 is composed of two subunits (HIF1α and HIF1β) from which HIF1α is regulated by hypoxia on the post-translational level (Marples et al., 2003). When five copies of HRE were linked to a minimal CMV promoter, hypoxia induced gene expression by 500-fold (Shibata et al., 2000).

Patterson et al. (2002) constructed a hypoxia-regulated expression vector encoding the cytochrome P450 reductase gene. When this vector was transfected into human fibrosarcoma cells the cytotoxicity of the alkylating nitroimidazole pro-drug RSU1069 increased 30-fold under hypoxic conditions.

It was mentioned earlier that the bioreductive drug tirapazamine (TPZ) had preferential cytotoxicity toward hypoxic cells. Cowen et al. (2004) prepared an adenoviral vector where the expression of the NADPH:cytochrome P450 reductase gene was regulated by HREs from

the lactate dehydrogenase gene. In a human tumour model where TPZ alone did not augment radiotherapy effects, they detected complete tumour regression when tumours were virally injected before treatment.

There are possibilities to construct dual, hypoxia- and radiation-induced promoters as well (Marples et al., 2003).

Modification of the normal tissue response

Radiation-induced early and late toxic effects might develop in about 5-10% of cancer patients undergoing radiation therapy. Toxicity to normal tissues is usually driven by the production of reactive oxygen species (ROS) such as superoxide radicals. ROS might be eliminated by radical scavenging enzymes (superoxide dismutases, SOD). Zwacka et al. (1998) cloned both the Mn-SOD and CuZn-SOD into an adenoviral vector and proved that the transduction of these genes into human lung epithelial cells reduced the level of apoptotic cells after irradiation by 50%. Epperly et al. (1998) demonstrated that the delivery of the Mn-SOD into the lungs of mice before radiation exposure substantially decreased the late effects in the lung.

CONCLUSION

The recent experimental and clinical data provided a large amount of excellent evidence that the combination of radiation therapy with gene therapy might be very useful for cancer patients. So far, three cancer gene therapy protocols reached clinical trial phase 4. Two of them received permission in China and the third one both in Europe and in USA. All of these protocols apply gene therapy with existing clinical treatment protocols including radiation therapy. Still, additional clinical investigations are necessary to prove the beneficial effects of these combinations.

ACKNOWLEDGEMENTS

This work was supported by a Hungarian NKFIH K124937 grant.

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