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Oncolytic adenovirus: preclinical and clinical studies in patients with human malignant gliomas

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1. Oncolytic adenoviruses: concept and rationale for their use as anti-glioma agents

During the decade of 1990 several efforts were made to overcome the suboptimal delivery of therapeutic genes to cancer cells. One of these strategies was the substitution of retroviral vectors for adenoviral vectors. Adenoviral vectors appeared to be easy to manipulate and very safe in vitro and in vivo. As a result, numerous pre-clinical studies were conducted using adenoviral vectors to transfer tumor suppressor genes or other therapeutic genes [1]. Eventually, the test of the adenoviral vectors in the clinical setting suggested that these vectors were not able to transduce enough number of tumor cells to result in a dramatic therapeutic effect[1]. Virology and molecular therapy laboratories were then focused on solving the “delivery problem”. One of the solutions was the generation of tumor selective oncolytic adenoviruses. These adenoviruses differ from the adenoviral vectors in the ability to replicate. The replication of the adenovirus within a tumor should theoretically multiply the input dose and cause a progressive spread of the virus with the potential to target every cancer cell in a given tumor.

The use of tumor selective oncolytic adenoviruses is particularly attractive for malignant gliomas. These tumors are normally constitutedof a single mass and almost never metastasize. Although these tumors displaystrong resistance to systemic therapies due to the existence of the blood-brain barrier, surgeons are able to reachthem to inject intratumorally the therapeutic adenoviruses using safe and fast procedures. In addition, the brain is an immune privileged organ because of the existence of the blood-brain barrier and a lack of cell-mediatedantigen drainage to the cervical lymph nodes[2]. These conditions are favorable for the replication and spread of the virus within the tumor.

Oncolytic adenoviruses are genetically manipulated human adenoviruses that acquired a replication phenotype in tumor cells, but show a more restricted phenotype in normal cells. Several features of wild type adenoviruses can be modified to acquire tumor replication properties[3]. The most frequently tested modifications involve the deletion of viral genes that interact with tumor suppressor genes, the modification of the tropism to infect cancer cells with more potency, and the inclusion in the viral genome of elements of transcription that are sensitive to transcription factors upregulated in cancer cells [1]. In this review we will focus on these aspects of virotherapy and vectorology, and we will describe in detail the history of Delta-24, Delta-24-RGD, ICOVIR and ONYX-015 as paradigms of these strategies.

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2. Adenoviral vectors targeting the Rb pathway

The development of glioblastoma multiforme involves progressive inactivation of several tumor suppressor genes including the retinoblastoma (*Rb*) tumor suppressor gene. Inactivation of the Rb protein is found in the majority of cancers, including at least 30% of malignant gliomas[4]. In addition, the majority of malignant gliomas exhibit disrupted Rb pathway, with the deletion of the locus encoding p16, p14ARF and p15 in more than 50% of the examined cases[4]. We constructed an adenoviral vector to transfer Rb protein to glioma cells lacking Rb expression[5]. In vitro, we observed that the Ad5CMV-Rb adenovirus carrying a 3.2-kb cDNA of the Rb gene transduced the exogenous protein very efficiently. Treatment of glioma cells with Ad5CMV-Rb resulted in the induction of a potent growth arrest, but not lack of viability. Thus Ad5CMV-Rb induces cytostatic but not cytocidal effect. However, this effect was enough to abolish the capacity of glioma cells to form tumors in nude mice. Although these findings provide direct evidence that inactivation of the retinoblastoma protein is a critical event in gliomas, further experiments in our laboratory could not demonstrate an antiglioma effect in vivo. Therefore, despite the fact that abnormalities in the Rb pathway are present in most gliomas including those expressing a wild type Rb protein[4], targeting this pathway by replacing of lost tumor suppressor activity through the transfer of *Rb* gene, or Rb-related genes such p16 is not sufficient to eradicate tumors in vivo[4]. This is because replication-deficient adenoviral vectors are unable to transfer the exogenous gene to sufficient number of cancer cells [6]. One potential solution to this conundrum is the generation of an oncolytic (replication-competent) adenovirus whose replication phenotype isrestricted to cancer cells with defects in the Rb pathway. This approach will potentially obviate the major hurdle of vector-based therapy: the need to infect the majority of the cancer cells with the input dose.

3. Oncolytic adenovirus targeting the Rb pathway: The Delta-24 prototype

To overcome the difficulties mentioned above and generate an oncolytic adenovirus, we combined the selectivity of the anti-cancer effect with a better delivery system and initiated the generation and characterization of a recombinant adenovirus encompassing a deletion of eight amino acids in the Rb-binding region of the E1A protein[7]. The E1A proteinissynthesized early after the infection and are required for viral replication to occur [8,9]. We hypothesized that a genetically modified adenovirus unable to bind and inactivate Rb would be able to replicate in gliomas that have disrupted Rb function, but not in normal cells. To select the region of E1A that needed to be deleted, we took advantage of meticulous and elegant workperformed in Harlow's laboratory[10]. Two well-described and exhaustively characterizedsegments of E1A are important for binding Rb [10,11]. Deletion of either region prevented the formation of detectable E1A/Rb complexes in vitro and in vivo[10]. For our study, we used these observations to develop a mutant adenoviruswith deletion of 24 nucleotides in E1A gene, termed Delta-24[7]. Testing Delta-24 in human glioma and other cancer cells revealed that the adenovirus was a powerful therapeutic agent in vitro and in vivo. Importantly, we also observed that the mutant E1A protein could not alter the cell cycle and kill non-cycling cells with wild-type Rb. We confirmed that the restoration of exogenous Rbin Rb-null cells render them resistant to cytopathic effectcaused by Delta-24 [7]. Perhaps of further clinical relevance, Delta-24 induced potent anti-cancer effect in glioma-bearing rodents and were more powerful tools than adenoviral vectors to treat gliomas by directly targeting the Rb pathway.

4. Enhancing the tropism of Delta-24: Delta-24-RGD

Although the anti-glioma effect of Delta-24 was remarkable, the anticancer effectwas not equal in every cell line. This was, at least, in part due to the inability of Delta-24 to efficiently infect cancer cells that do not express the native receptors for adenoviruses. It is now accepted that

the ability of adenoviruses to infect tumor cells depends onanchorage to the coxsackie-adenovirus receptor (CAR) [12] and that the internalization of the adenovirus depends on the expression of RGD-related integrins. Therefore, glioma cells expressing low levels of CAR presented a barrier to an efficient infection by Delta-24 [13]. Once this obstacle was recognized, we undertook an effort to modify Delta-24so that it was capable of infectingtumor cells via CAR-independent mechanisms. Because internalizationof the virus into host cells is mediated by a secondaryinteraction between RGD motifs on penton base protein loopsand integrins αvβ3 and αvβ5[14], Delta-24 was modified to targetintegrins on the surface of cancer cells as its primary receptor. This was achieved by inserting the sequence of a previously identifiedpeptide ACDCRGDCFCG (RGD-4C), which binds strongly to the αvβ3 and αvβ5integrins [15,16], intothe HI loop of the fiber knob protein to transform Delta-24 into Delta-24-RGD [17]. Our studies showed that infection of U-87 MG cells (a low-CAR-expressing line) with AdGFP-RGD resulted in approximately six times more GFP-positive cells than infection with AdGFP. Accordingly, Delta-24-RGD was more cytopathic to low-CAR-expressing glioma cells than its predecessor, Delta-24. In the xenografted mice, intratumoral injection of Delta-24-RGD was associated with extended survival and, importantly, more than half of the treated mice survived more than 4 months. Pathologic examination of the brain of these animals showed the complete suppression of the tumors. Based on these results, it was cautiously proposed that the antitumor activity of Delta-24-RGD has the potential to be a useful tool for the treatment of patients with malignant gliomas. Delta-24-RGd is now being tested in a Phase I clinical trial in patients with recurrent malignant gliomas.

5. Delta-24 and chemotherapy

The study of the history of cancer therapy reveals that eradication of cancer could be better achieved using a combination of therapies. Because chemotherapy and virotherapy has weaknesses of their own, others and we postulate that both strategies can be combined to obtain a synergistic anti-glioma effect. There are a series of rationales to base the combined strategy. But in agreement with the format of this review, we will focus on the ones that we have tested in our laboratories. Because Delta-24 infection caused human glioma cells to accumulate in the S phase and it was reported previously that replication of adenoviruses results in the increased expression of topoisomerase I[9], we examined whether Delta-24 could be combined with topoisomerase I-targeted drugs[18]. In this work, we showed that the sequential administration of Delta-24 (given first to increase the amount of the molecular target to the chemotherapy)and then CPT-11 to glioma cellsenhanced the effect of the drug in vitro, and importantly, without diminishing the killing potency of the oncolytic adenovirus. Studies in rodents confirmed what we observed in vitro and revealed that the administration of Delta-24 followed by the systemic administration of CPT-11 resulted in significantly prolonged animal survival[18].

More spectacular results were obtained using the combination of Delta-24-RGD and temozolomide. Currently, the majority of glioma patients are treated with the drug. Temozolomide is more efficacious in patients with malignant gliomas that do not express the DNA repair enzyme O(6)-methylguanine-DNA methyltransferase (MGMT), due to silencing of the MGMT promoter[19]. Because MGMT reverses the DNA damage caused by temozolomide and thus renders cancer cells resistant to this agent, the patients with gliomas expressing the enzyme are refractory to the therapy[19]. Based on the concept that adenoviruses required the inactivation of key DNA repair genes in order to replicate[20], we hypothesized that the oncolytic adenovirus Delta-24-RGD could down-modulate the MGMT-mediated resistance and thus be successfully combined with temozolomide. Indeed, we observed in cultured cells that Delta-24-RGD infection down-regulated the RNA levels of MGMT[21], inducing an effect similar to the methylation of the MGMT promoter. Importantly, we

demontrated that the interaction between adenoviral E1A protein and p300 was required to induce silencing of the MGMT gene. Finally, in vivo studies revealed that the combination of Delta-24-RGD and temozolomide significantly prolonged the survival of glioma-bearing mice [21]. These studies indicate that it is rational to combine oncolytic adenoviruses and temozolomide in glioma therapy. Currently we are planning to testthe combination in glioma patients.

6. Delta-24 and cancer stem cells

The brain tumor stem cell hypothesis proposes the existence of multipotent glioma cells, characterized by the expression of normal stem cell markers and by their capacity for self-renewal and asymmetric differentiation, constitute the tumor-initiating population of cells [22–26]. Brain tumor stemcells are a key cellular target for the development of new therapies because they are believed to be resistant to radiation and chemotherapy, and may therefore be responsible for the recurrence of gliomas after therapy[27,28]. Relevant to this review, susceptibility to adenoviral infection and replication were recently examined [29]. To test the anti-cancer effect of Delta-24-RGD in brain tumor stem cell models. We first isolated neurosphere-forming cells from fresh surgical specimens of glioblastoma multiforme [29]. When cultured in growth-factors enriched media, these cells showed self-renewal and expressed CD133[29]-a cancer stem cell marker in brain cancer and in other solid tumors[22, 30,31]. The cells were alsocapable to differentiate to neurons and astrocytes when the culture medium was depleted of growth factors[29]. The cell lines initiated new tumors when transplanted into the brains of mice. We examined in these cells the levels of Rb and p16INK4a (p16) proteins, whose expression is mutually exclusive in gliomas [32,33]. We found that one cell line did not express Rb but express p16, and the other three expressed Rb but did not express p16 [29]. Once confirmed the disruption of the Rb pathway in these cell lines, we examined the expression of adenovirus receptors. To our surprise, we found that brain tumor stem cells expressed high levels of CAR and, as we expected, high levels of integrins[29]. Infectivity of these cells, thus, did not seem to be a barrier for adenovirus-based therapy. In addition, the xenografts derived from brain tumor stem cells recapitulated the features of primary malignant gliomas and showed a higher infiltrative phenotype than other xenograft models such as the one derived from U-87 MG cells[29,34]. Thus, the phenotype and natural evolution of the tumors generated with brain tumor stem cells are more similar to primary tumors in human patients and cause more difficulties to conventional therapy than models based on conventional established glioma cell lines. Then, we tested the therapeutic efficacy of Delta-24-RGD in the brain tumor stem cell xenograft model and we found that the treated animals survived longer than those in the control group[29]. In summary, adenovirus-based therapy seems to be a highly effective strategy to target and eliminate brain tumor stem cells.

7. Delta-24 and autophagy

Kondo's group reported that oncolytic adenoviruses triggered autophagy in glioma cells [35]. Moreover, they demonstrated that there was an inhibition of the pohosphorylation of one of the downstream targets of mTOR and suggested that adenoviruses caused autophagy in infected cells trough inactivation of the AKT/TOR pathway[35]. Later it was published that Delta-24-RGD induced autophagy in glioma cells in vitro and in vivo [29]. Importantly, key autophagy players were upregulated during adenoviral infection. Thus after Delta-24-RGD infection, the level of the protein complex formed by Atg5 and Atg12 was found dramatically increased [29]. The ATG5/ATG12 levels were also found high in xenografts treated with Delta-24-RGD [29]. The meaning of autophagy during adenoviral infection is unclear. It could be a feature of the innate immune response against the virus or one of the ways utilized by the cell to present viral antigens [36]. It could also be possible that adenovirus subvert the autophagy pathways and exploit this catabolic process for its own benefit [37]. In this model, the virus will take

advantage of the ATP and aminoacids generated during the autophagy, recycling long-lived proteins and organelles[37]. In addition to these possibilities, we have proposed that autophagy may be part of the adenovirus-induced cell lysis [37]. Serious deterioration of the cytoplasmic architecture due to unregulated formation of excessive autophagosomes, as well as the induction of a metabolic catastrophe induced by the hectic anabolic process that permits adenovirus replication, can cooperate to trigger autophagic cell death as part of the final stage of the adenoviral life cycle[37]. We have to conclude, however, that at the moment of writing this review, in the topic of adenovirus and autophagy, every study will open more questions that will result in ultimate answers.

8. Delivery of adenoviruses using human mesenchymal stem cells

Several groups have provided evidence suggesting that stem cells are useful deliveryvehicles for brain tumor therapy. Two main systems are the frontrunners in the field: neural stem cells and human mesenchymal stem cells (hMSC). First came the evidence that, after intracranial injection,neural stem cells have a tropism for brain tumors [38]. Other group showed that neural stem cells engineered to deliver interleukin-12 or tumor necrosis factor–related apoptosis-inducing ligand inhibited the tumor growth[39,40]. Because the difficulty in the isolation of neural stem cells from patients, hMSC appeared soon as an alternative to neural stem cells. hMSC are easily obtained from patients and autologous transplantation, which obviates immunologic incompatibilities, is possible[41]. The rationale for using bone marrow–derived stem cellsis based on the tenet that circulating stem cells are recruited from the blood into peripheralsolid organs under stress or injury [42–46]. Using an intracranial model of gliomas, Lang's laboratory showed that hMSCs had atropism for human gliomas after intravascular and local delivery, and that this tropism can be exploited therapeutically by using the cells as vehicles for adenoviral vectors [47]. In another study, Lesniak's laboratory examined the feasibility of using hMSCs as vehicles for replication-competent oncolytic adenoviruses[48]. These investigators observed that virus-loaded hMSCs effectively migrated in vitro and released adenoviruses that infected U-87MG glioma cells. When injected away from the tumor site in vivo, hMSCs migrated to the tumor and deliveredthe viral payload. In a more recent work, Germano's laboratory demonstrated that embryonic stem cell (ESC)-derived astrocytes could be used to deliver pro-apoptotic genes to malignant glioma cells[49]. In this study, ESC-derived astrocytes conditionally expressing TRAIL were injected into glioma xenografts. The authors observed a significant decrease in tumor volume after the injections of the cell vehicle. Importantly, they found that, several days after injection, the majority of the tumors had undergone severe necrosis[49]. The three systems discussed here are just samples of several cell types that could be potentially used to deliver therapeutic agents to malignant gliomas. Preclinical data are offering strong evidence that the “Trojan Horse” strategy of delivering molecular and biological therapies using tumorotropic cells could be useful in the treatment of human patients and should propel the development of clinical trials to testtheir capability to deliver oncolytic adenoviruses intratumorally and systemically.

9. The third generation of Delta-24 vector: ICOVIR

Although Delta-24-RGD could be considered a safe therapeutic system when administered intratumorally, high levels of E1A could result in toxicity in liver and other organs when it is delivered systemically. To diminish E1A-mediated toxicity, Alemany's laboratory [50] engineered an oncolytic adenovirus termed ICOVIR that encompasses the signature modifications in Delta-24 (partial deletion of E1A), Delta-24-RGD (RGD-4C insertion in the fiber knob) and the inclusion of E2F1 responsive elements as ectopic promoter for E1A [51]. In this construct, therefore, E1A expression is regulated by the Rb pathway and is silenced in normal, fully differentiated, postmitotic cells. Consistent with this hypothesis, ICOVIR displayed a formidable therapeutic index, broader than Delta-24-RGD, when administered

intravenously [50]. Importantly, the anti-glioma effect of this construct could be amplified by combination with chemotherapy. Thus, combined administration of ICOVIR with rapamycin showed dramatic improvement in the survival of glioma-bearing mice [52]. It is interesting to determine whether the molecular basis of the synergy reside on the activation of autophagy by rapamycin-mediated inhibition of mTOR.

10. Adenoviral vectors targeting the p53 pathway

P53 is one of the tumor suppressor genes most frequently found inactivated in human cancers [53]. As a result, several strategies have been tested to trigger a potent p53-mediated apoptosis in cancer cells. Using adenoviral vectors, our group showed that transfer of p53 to glioma cells expressing mutant p53 protein resulted in apoptosis[54]. Based on these data, a clinical trial was initiated to test the efficacy of adenovirally-mediated p53 in recurrent gliomas. The clinical trial revealed that using adenoviral vectors to transfer p53 was safe and that the adenovirus transferred a functional p53 protein to glioma cells[55]. However, the examination of the treated tumors indicated that the transduction of p53 was found in a minority of tumor cells that were surrounding the injection site [55].

11. Oncolytic adenoviruses targeting the p53 pathway: ONYX-015

McCormick group reported an oncolytic adenovirus that acquired a replication phenotype depending on the p53 status in cancer cells[56]. Theirwork caused a tremendous revival of virotherapy. The mechanistic rationale behind the ONYX-015 construct was the interaction of the adenoviral protein E1B-55k with p53 [57]. E1B-55k protein binds to and inactivates p53 in order to prevent the infected cell to commit altruistic apoptosis and thus impeding viral replication and the spread of the virus to the neighbor cells [57]. In their study, the authors utilized an adenovirus depleted of E1B-55k protein and found that the replication phenotype was fully acquired in cells in which p53 was mutant, but not in cells expressing a wild type p53 [56]. Based on these data, ONYX 015 was moved to the clinic and was tested in several cancers [58]. In brain tumors, ONYX-015 was tested in the setting of multicentric clinical trials. In patients with malignant gliomas, the tumor was removed first and then ONYX-015 was injected into several sites in the walls of the cavity. The study demonstrated that ONYX-015 was safe, but did not show significant anti-tumor activity[59]. The mechanism of the tumor selectivity of ONYX-015 has been reexamined. Currently, the role of the p53 protein in its tumor selectivity is not considered to be the main factor [60]. Although ONYX-015 has not shown therapeutic efficacy as a single anti-cancer agent, its combined administration with chemotherapy resulted in encouraging effect in patients with head and neck cancer [61]. After a Phase III clinical trial in China[62], an E1B-55k mutant adenovirus, mechanistically similar to ONYX015, has been approved by the Chinese FDA for its use in patients with head and neck cancer in combination with chemotherapy.

12. Summary and conclusions

Replication-competent oncolytic adenoviruses hold the promise to be part of a greater and more sophisticated armamentarium of future therapies for gliomas. Several features of these biologic agents render them more advantageous than adenoviral vectors. The fact that these viruses target and replicate in brain tumor stem cells is a key point to include them in combination with other therapies to which the cancer stem cell population are resistant. Several problems identified in the past still remain as determinants of a successful therapy. These include the lack of a relevant animal model to test the immune response of the host against the virus.In addition, a deep analysis of the immune system in patients treated with these agents is still pending. However, in the preclinical setting, combination of oncolytic adenoviruses with chemotherapy has consistently showed dramatic anti-glioma effect. The discovery of the mechanisms underlying the resistance to chemotherapy (such as the methylation and silencing

of the MTMG gene) and the action of certain drugs (such as the induction of autophagy by rapamycin) are paving the way for developing more rational combinations of virus and chemotherapy. The monitoring of clinical trials will also benefit enormously from the development of noninvasive imaging methods that will allow the real time study of the kinetics of viral replication in vivoto evaluate the effect in patients. Perhaps, one future direction will be the use of adenovirus in which viral proteins that trigger the anti-viral immune response will be substituted by cancer specific antigens and thus the virus will combine the benefits of virotherapy and immune-genetherapy.

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