Review Article

Biologic therapy and gene therapy in the multimodality treatment of malignant pleural mesothelioma

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Abstract: The last years have witnessed an abrupt paradigm shift in cancer treatment owing to the discoveries concerning the relationships between the immune system and neoplastic cells. In the field of malignant mesothelioma, which, despite painstaking efforts, remains an incurable form of cancer, the researchers' attention has been seized by a variety of new biologic approaches, including both viral gene therapy and active immunotherapy. The former is meant to induce programmed cell death by introducing a specific gene in the target cell, this gene encoding a specific protein with anticancer activity. Active immunotherapy, on the other hand, tires to induce an active response of the immune system, whose surveillance may be easily dodged by cancer cells. In fact, this mechanism seems to play an important role in the development, growth and diffusion of malignant mesothelioma which easily manages to hinder the immune response. A thorough understanding of the relationships existing between mesothelioma and immune system is the basis for the success of those immune therapies, which are showing promising results in the preclinical setting, especially when combined with other approaches, such as cytoreductive surgery.

Keywords: Malignant mesothelioma; immune therapy; virotherapy; cancer gene therapy; cytoreductive surgery

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Introduction

In recent years, immunotherapy has become an important tool in the treatment of advanced-stage pleural mesothelioma (1), especially as second line therapy. At the same time, however, many studies have been testing immunotherapy in combination with surgery, mainly in the experimental setting (2). Various animal models had been developed as yet, employing direct administration of immunomodulators in the tumor environment, or viral probes in order to infect the neoplastic cells. Immunomodulators, such as interleukins, are expected to prompt an activation of those cells naturally despatched by the immune system within the tumor, but, for unknown reasons, unable to start a proper defensive response (passive immunotherapy). The active models, which entail a viral infection limited to target (neoplastic) cells, are becoming more and more popular. Viruses may be used for their specific oncolytic activity (virotherapy), or in order to transfer a specific protein-encoding gene with antineoplastic activity within tumor cells (viral gene therapy). Furthermore, with the aid of viruses, researchers have been recently trying to force the neoplastic cells to expose well-defined molecular targets to the immune system (active immunotherapy) (3).

Many different biologic targets have been found, and thanks to translational research, tested in experimental settings, both *in vitro* and *in vivo*. The results, although referring to small groups of animals and, rarely, to human cohorts, seem to suggest a possible "fourth tenet" in the multimodality treatment of malignant mesothelioma,

traditionally hinged upon chemotherapy, surgery and radiotherapy (4).

Results

Passive immunotherapy

In 2007, Lucchi *et al*. (5) introduced a sort of immunotherapy in the multimodality treatment of malignant mesothelioma in a phase II trial. A total of 49 patients with stages II-III pleural mesothelioma according to International Mesothelioma Interest Group (IMIG) staging system underwent preoperative intrapleural (IP) IL-2 (18×10^6 UI/day for 3 days) followed by Pleurectomy/ Decortication. The treatment schedule then included IP epidoxorubicin 25 mg/m^2 for 3 days and 5-7 days after surgery followed by IP IL-2 18×10^6 UI/day for 3 days. Chemotherapy with Cisplatin 80 mg/m^2 on day 1 and gemcitabine 1,250 mg/m^2 on days 1 and 8 was then administered for 3-6 courses. The immunotherapy continued with subcutaneous administration of IL-2 3×10^6 UI/day on 3 days per week as long as possible. The treatment was averagely well tolerated. The main side effect of IL-2 administration consisted in fever (during preoperative administration), and fever accompanied by eosinophilia during the long-term subcutaneous course. The proposed treatment resulted in a good median survival (26 months). The 2- and 5-year actuarial survival rates were 60.2% and 23.3%, respectively. Performance status according to ECOG results the only factor to really affect survival. The experience of Lucchi and colleagues represents a good example of passive immunotherapy. However, other modulators had been tested before. In a pioneering experience (6), passive immunotherapy was combined with chemotherapy, and the following compound approach was developed: cisplatin (25 mg/m^2 4 times weekly), interferon-α (5 mU/m^2 s.c. 3 times weekly), and tamoxifen (20 mg orally twice a day for 35 days). The authors had 36 patients undergoing the treatment. Furthermore, in ten patients it was scheduled in an adjuvant fashion, following "maximal cytoreduction" surgery. A partial radiological response was appreciated in 19% of patients. Toxicity profile was acceptable (4% grade III/IV), with one patient dying from myocardial infarction. Median survival responders were 14.7 months, whilst nonresponders survived averagely 8 months. Median survival for the entire group was 8.7 months. Preoperative size, platelet count >360,000/mL, and non-epithelial histology associated with lower survival.

Another indirect way which has been experimented implied the use of monoclonal antibodies. In particular, blockade of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), a T-cell surface antigen that plays an important role in their activation, entails a delayed tumor growth in various experimental models. A recent study (2) has shown the potential of CTLA-4 specific monoclonal antibodies, especially when combined with radiotherapy, in slowing the growth of experimental murine flank mesothelioma. Interestingly, in mice bearing two tumors, the irradiation of only one tumor provoked growth delay also in the untreated tumor, owing to the activation of tumor specific T cells, whose effect exerted systematically (so called abscopal effect).

Gene therapy and active immunotherapy

Recently, many immunologic/biologic treatments have been tested in combination with surgery, both with an adjuvant and neoadjuvant intent, in particular in pre-clinical models. Fisher and colleagues (7) developed a murine model of mesothelioma, in which injecting in the flank clonal mesothelioma cells induced an etherothopic tumor. Those cells were created from AB1 clonal cells then transfected with PR8 influenza virus hemagglutinin (HA) gene, in order to express the surface molecule HA (5×10^5 AB1-HA cells in a total volume of 100 µL PBS solution), that should act as a target. At the same time, a viral vaccine was created to stimulate an immune response towards the HA surface protein of tumor cells. Subjects undergoing vaccination followed by surgery showed a decreased tumor burden and a prolonged survival when compared to those treated only with surgery. To note, the mice receiving the virotherapy developed a tumor specific immune response, fostered by CD8 lymphocytes.

Acuna and colleagues developed an experimental model to prove the anticancer activity of viral vectors against mesothelioma cells (8). The virus employed was a vaccina virus, with deletions of the viral thymidine kinase (TK) and vaccina growth factor (VGF) genes (double deleted vaccina virus, vvDD). The model encompassed a first step consisting in direct infection of two lines neoplastic mesothelioma cells (AC29 and AB12) *in vitro*. Then, an *in vivo* orthotopic model was created by seeding of neoplastic cells AC29 and AB12 in the peritoneal cavity of immunocompetent mice (CBA/J and BALB/mice). In order to identify viral infection and replication a reporter gene was incorporated in the viral DNA (expression of a red fluorescent protein). Viral vectors were injected directly

in the peritoneal cavity at various times: after 72 h from neoplastic seeding as a model for microscopic disease, after 10 days (at that time all mice would present macroscopic disease) and after surgical removal of bulky tumor nodules (cytoreductive surgery allowed removal of 53.8% to 60% of gross disease). Survival of mice was then evaluated and compared to controls. vvDD treatment resulted in specific cytopathic activity *in vitro* against neoplastic cell cultures at a significantly lower multiplicities of infection (MOI) when compared to control cells (Fibroblasts; P<0.001). In the *in vivo* model virus administration after 72 h from malignant cell seeding resulted in increased median survival by 10 days (P=0.001). The impact on survival of viral administration was still evident when the administration took place after 10 days. In particular, the AB12 mesothelioma-bearing mice, vvDD-SR-RFP significantly improved median survival by 9 days compared with the vehicle-treated controls (P<0.001). On the other hand, the impact of viral therapy was less evident with AC29 cell line tumors (P=0.088). Nonetheless, when tested as "adjuvant" after cytoreductive surgery, virotherapy did not determine an increase in survival compared to virotherapy alone.

Intratumoral virus administration has been also employed in combination with standard chemotherapy in experimental settings. The main belief at the basis of this combined therapy relies upon a 2-steps mechanism: immune cells activates against specific tumoral antigens, provided by virus infection of tumor cells (primer), thereby forming an immunologic memory, then, systemic chemotherapy provides a great amount of circulating tumoral antigens (derived by cytolysis), providing an immunologic boost and a subsequent enhanced response against residual tumor cells. In 2011, Fridlender and colleagues (9) developed a murine model in which large flank (xenograft) tumors of AB12 cells were treated with intratumoral administration of one dose of INF producing Adenovirus followed by chemotherapy (cisplatin + gemcitabine). Virotherapy alone could slow down tumor growth, although tumor regression was not observed. On the other hand, virotherapy in combination with subsequent chemotherapy resulted in tumor shrinkage (tumor size was significantly smaller at the end of treatment, P<0.05). This effect on tumor growth kinetics depended on the development of a strong immune response. The primer induced T- memory cells against defined neoplastic antigens, those cells were then activated by the boost. Specific T-cells were increased by four to tenfold after virotherapy when compared to unchallenged controls (P<0.05). Chemotherapy boost activated those memory cells, thus leading to augmented circulating $CD8^+$ cytotoxic (oncolytic) lymphocytes, intratumoral $CD8^+$ lymphocytes (twentyfold when compared to controls, P<0.05). Chemotherapy diminished counter regulatory immunological mechanisms as well, by stopping the increase of inhibitory cells, and increased the ratio of antitumorigenic (M1)/protumorigenic (M2) macrophages. Gemcitabine augments leukocyte trafficking in the tumor tissue and up-regulates NF-κB in tumoral cells.

Other authors employed multiple viral vectors in order to achieve a synergic effect. Watanabe and colleagues (10) developed a murine model (human mesothelioma cell lines H2052 and H2452 forming pleural mesotheliomas in athymic nu/nu mice) in which a modified adenovirus would knock out telomerase activation in the target cells. Cytopathic power was tested *in vitro* and then confirmed *in vivo*. In order to augment the antitumoral effect of the virus directed against telomerase activity, another virus, codifying heparanase, a protease that degrades heparin sulfate, was created and administered (Ad-S/hep). This "adjuvant" virus was meant to disrupt the extracellular matrix within tumoral microenvironment, enhancing the diffusion of the viral vector within the tumor bulk. Co-infection with OBP-301 virus, stopping telomerase activity, and Ad-S/hep virus, disrupting extracellular matrix resulted in a more profound antitumoral activity both *in vitro* and *in vivo* (reduction of tumor weight on day 43 when compared to mice infected only with Ad-S/hep; P<0.05). In 2009, Ampollini *et al*. (11) developed an rat model to evaluate the role of immunomodulation of the Innate Immunity trough the stimulation of a specific receptor, called Tol-like receptor 9 (TLR-9). The triggering of TLR-9 directly activates human B-cells and macrophages leading to activation of natural killer cells via Il-6, IL-12 and TNF-α production. TLR-9 was stimulated by an artificial unmethylated sequence of DNA (similar to those motifs of viral or bacterial DNA that are the natural ligands for TLR family) called CpG28. In the experimental environment, 24 rats affected by experimental pleural mesothelioma were treated with pleural resection and pneumonectomy (to simulate extrapleural pneumonectomy) followed by CdG28 inoculation alone (6 cases), Cisplatin-imbued fibrin glue alone (6 cases), or combination of those therapies (6 cases), the remainder was left untreated after surgery and acted as control. Primary end point was the volumetric evaluation of tumor recurrence according to various attempted post-surgery therapies. Both cisplatin-fibrin glue and cisplatin-fibrin + CpG significantly reduced

the volume of tumor recurrence, when compared to control and to immunotherapy alone (P=0.004 and P=0.004). Activated $CD8^+$ cells were significantly more represented when CpG was part of the therapy. Other immune cell populations were not significantly affected. In conclusion, the introduction of immunotherapy on TLR-9 did not affect the tumor recurrence volume. The authors believed that this could be related to the short observation time after treatment (6 days), which didn't allow a proper immune anticancer response to ensue.

In 2007, Adusumilli and colleagues (12) evaluated the synergic effect of the combination of radiotherapy and immunotherapy in an animal model (athymic mice, flank tumor from JMN mesothelioma cell line). They employed an oncocytic herpes virus (NV 1066) with deletion of a gene encoding for ICP 34.5, a protein that prevents the arrest of protein synthesis within the infected cell (a common defense mechanism of infected cells to snuff off viral replication). This gene deletion allows a safe use of NV 1066 as a vector. The function of ICP 34.5 could be restored in damaged tumor cells. Under particular stimuli (DNA damage) they produce a protein (namely GADD 34) that can vicariate the functions of ICP 34.5, thereby restarting the viral replication and the resulting cytotoxic effect. After a first round of *in vitro* evaluation, the subjects were then randomized into four groups: no treatment (control group), radiotherapy alone (2.5 Gy), virotherapy alone (single intratumoral injection of NV 1066 herpes virus) and RT followed by intratumoral injection of 10^7 PFU NV1066 (24 h later). The combination therapy resulted in a statistically significantly better cytotoxic effect than single-agent therapy, as witnessed by tumor volume at day-12 (P=0.01 on day 21 by *t*-test).

Kruklitis *et al.* (13) tested the efficacy of INF-β encoding Adenovirus. The authors injected the virus in AB12 mesothelioma cells tumors, established in the flank of BALB/c mice. The efficacy of the virus decreased as the volume of the tumor at the moment of injection grew. In particular, virus administration to those tumors smaller than 200 mm^3 resulted in complete response. When evaluating the antineoplastic immune response by isolation of antitumor specific $CD8^+$ T lymphocytes (cytotoxic T lymphocytes, CTL), immune cells harvested from mice that were treated at a low tumor burden showed the capability of stopping neoplastic growth. When mixed with AB12 tumor cells and then injected in naïve mice, these cells showed a significant anticancer potential. The production of a specific line of CTL is associated to the extent of inflammatory cell clusters within the tumor. The scarcity of inflammatory infiltration within large tumors seemed to impair the development of an adequate CTL response. The authors then tested the hypothesis that immunotherapy could prevent relapse after large tumor debulking and proved that neoadjuvant virotherapy, administered 3 days before surgical resection, resulted in a delay of tumor recurrence and in prolonged survival (mean recurrence time: 19 *vs.* 6 days, P<0.01, 58% *vs.* 11%, P<0.01).

Conclusions

The development of experimental mesothelioma models either with the aim of harnessing the activation of a specific immune response or introduce a gene owning anticancer activity has shown promising results in terms of both tumor burden reduction and survival. Furthermore, a possible synergic action with cytoreductive surgery has been clearly proven, resulting in a promising application of those "alternative" biologic therapies in the multimodality treatment of mesothelioma.

At the same time, those models have unravelled the complexity of the interaction between the tumor and the immune system, depicting the peritumoral environment as a highly active milieu, where many different type of immune cells act in a both antineoplastic and pro-neoplastic way (14). Mesothelioma displays a powerful "anti-immune" activity, fostered by myeloid-derived suppressor cells, macrophages and $CD4^+$ cells (15). A thorough understanding of the immunologic landscape housing the tumor will certainly help us develop tailored treatments with lesser side effects than those already observed in the clinical setting. The acquired knowledge will then form the basis for the combination of immune therapy with other approaches, in particular with surgery.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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