Curative Potential of GM-CSF-Secreting Tumor Cell Vaccines on Established Orthotopic Liver Tumors: Mechanisms for the Superior Antitumor Activity of Live Tumor Cell Vaccines

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Key Words
Granulocyte-macrophage colony-stimulating factor (GM-CSF) • Tumor cell vaccine • Live vaccine • Irradiated vaccine • Orthotopic liver tumor

Abstract
In preclinical studies, tumor cells genetically engineered to secrete cytokines, hereafter referred to as tumor cell vaccines, can often generate systemic antitumor immunity. This study investigated the therapeutic effects of live or irradiated tumor cell vaccines that secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) on established orthotopic liver tumors. Experimental results indicated that two doses (3 × 10^7 cells per dose) of irradiated tumor cell vaccines were therapeutically ineffective, whereas one dose (3 × 10^6 cells) of live tumor cell vaccines caused complete tumor regression. In vivo depletion of CD8+ T cells, but not natural killer cells, restored tumor formation in the live vaccine-treated animals. Additionally, the treatment of cells with live vaccine induced markedly higher levels of cytotoxic T lymphocyte activity than the irradiated vaccines in the draining lymph nodes. The higher levels of cytokine and antigen loads could partly explain the superior antitumor activity of live tumor cell vaccines, but other unidentified mechanisms could also play a role in the early T cell activation in the lymph nodes. A protocol using multiple and higher dosages of irradiated tumor cell vaccines also caused significant regression of liver tumors. These results suggest that the GM-CSF-secreting tumor cell vaccines are highly promising for orthotopic liver tumors if higher levels of immune responses are elicited during early tumor development.

Introduction
Hepatocellular carcinoma (HCC) is a common tumor worldwide, and the most common cancer in Taiwan. The annual number of mortalities from HCC worldwide is estimated at 1,250,000 [16] and in Taiwan it is around 4,000 [7, 29]. In about 90% of cases, this malignant tumor develops on a background of chronic hepatitis and/or cirrhosis [10]. Surgical resection or orthotopic liver transplantation are potentially curative, but are most effective in patients with small and localized HCCs [34, 47]. Other locoregional therapies, e.g. percutaneous ethanol injection [31] or transcatheter arterial embolization [41], are mainly performed with palliative intent; whereas conventional chemotherapy or radiotherapy is ineffective for...
HCC [17, 28, 52]. The survival rate after the onset of symptoms is generally low, creating an urgent need for new therapeutic strategies.

Numerous experimental and clinical data demonstrate that tumor cells engineered to produce cytokines, namely tumor cell vaccines, have significantly increased immunogenicity and can induce antitumor immune responses and consequent tumor rejection [14]. The rationale for such an approach has been discussed extensively [11, 40] and lies in the recruitment of inflammatory cells, in response to the local secretion of cytokines that can partially destroy the tumor cells. The tumor antigens released from the dead tumor cells can be taken up by dendritic cells (DCs) that travel to draining lymph nodes, where they encounter and activate naïve T lymphocytes, thus generating systemic, and probably long-lasting, immune responses. Although different cytokines can recruit different inflammatory cells with different kinetics [37], the end results in generating systemic immunity are generally similar. Among the various cytokines that have been adopted to stimulate immune responses against different tumors [37, 46], granulocyte-macrophage colony-stimulating factor (GM-CSF) is one of the most potent, and its use in tumor cell vaccines has achieved some success in animal tumor models [2, 13, 33, 45]. The key to the role of GM-CSF as an immunomodulator is its ability to recruit and activate functional antigen-presenting cells (APCs) [8, 19, 49] such as DCs, the most potent activators of T cells [3, 18].

Previous animal studies demonstrating the efficacy of immunotherapeutic strategies on HCCs have mostly examined extrahepatic sites for tumor formation, particularly in the subcutaneous (s.c.) tissue [5, 15, 21, 53]. Although inoculation and monitoring of such sites are easier, the tumors may differ immunologically from those occurring within the liver. s.c. tumors may exhibit high inherent immunogenicity due to the presence in this localization of high levels of DCs, which are very efficient at presenting tumor antigens and eliciting immune responses [38, 42]. On the other hand, the liver is speculated to promote immunologic tolerance to foreign antigens [26, 43], and has been demonstrated to attract activated CD8+ T cells undergoing antigen-induced apoptosis [12, 25, 30, 36]. The mechanisms underlying these phenomena have not been well elucidated, and thus, the successful generation of antitumor immunity against s.c. tumors does not necessarily indicate the same efficacy against orthotopic tumors. Therefore, an orthotopic HCC model is the preferred experimental setting for analyzing the effects of tumor cell vaccines in a realistic microenvironment [4, 39, 42].

The tumor cell vaccines injected in animal models or human clinical trials are mostly irradiated to avoid their proliferation in vivo, but retain the ability to secrete cytokines for a short period of time. This phenomenon seems sufficient to mount an antitumor immunity capable of preventing the outgrowth of a subsequent challenge of parental untransduced cells. However, the immunity is often less effective in a therapeutic setting, that is when tumors are already established [40]. Some previous studies have demonstrated that the immunizing effect of live (replicating) tumor cell vaccines was superior to that of irradiated cells [1, 27, 50], but the mechanism for this remains unclear. Therefore, clarifying the mechanisms underlying the superior effects of live tumor cell vaccines is worthwhile, as it could possibly facilitate the design of a better, rationale-based strategy of immunotherapy for advanced tumors.

This study established orthotopic liver tumors in syngeneic animals and compared the curative potential of live and irradiated tumor cell vaccines. Significantly different therapeutic effects were observed on liver tumors for these two types of vaccine. Further mechanistic studies revealed that high levels of GM-CSF and antigen loads provided by live vaccine cells, plus some other unknown mechanisms, probably caused strong T cell activation in the draining lymph nodes and resulted in early and sustained T cell infiltration in the tumor regions. This work also demonstrated that the low immunogenicity of irradiated tumor cell vaccines could be compensated for by multiple injections of higher dosages of irradiated vaccines.

**Materials and Methods**

**Cell Lines and Vector**

GP7TB is a cell line derived from chemically transformed hepatic epithelial cells in a Fischer 344 rat [9, 35, 51]. The cells were maintained in Dulbecco's modified Eagle's medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biological Industries, Israel). NFS-60 is a murine myelogenous leukemia cell line derived from an NFS/N mouse and often used in GM-CSF bioassay [22]. The cells were maintained in RPMI 1640 medium supplemented with 10% FCS in the presence of 1 U/ml recombinant mouse GM-CSF (R&D, Minneapolis, Minn., USA).

A bicistronic retroviral vector, containing a mouse GM-CSF cDNA at the first cistron and a neo+ gene at the second cistron that is translated via internal ribosome entry sequences [23], was used to transduce GP7TB cells following previously described procedures [23]. The stable GM-CSF-transduced GP7TB cells (designated as GM/GP7TB) and the control vector-transduced GP7TB cells (designated as S2/GP7TB) were maintained in a selective medium containing G418 (0.8 mg/ml; Sigma, St. Louis, Mo., USA).