Biotherapy 3: 55–64, 1991.

Liposomal delivery of biological response modifiers to macrophages

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Received 6 March 1990

Key words: immunomodulators, liposomes, macrophage, macrophage activation, metastasis

Abstract

Macrophages activated to the tumoricidal state can recognize and destroy neoplastic cells and leave normal cells unharmed. Systemic activation of macrophages can be achieved by the intravenous administration of liposomes containing various immunomodulators. Much like any particle, liposomes are cleared from the circulation by phagocytic cells. This passive but specific targeting of immunomodulators to macrophages results in their activation for *in vitro* and *in vivo* lysis of tumor cells that can be resistant to conventional therapies.

Abbreviations: MAF: macrophage-activating factor; MDP: N-acetylmuramyl-L-alanyl-D-isoglutamine; MLV: multilamellar vesicle; MTP-PE: N-acetyl-muramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1′,2′-dipalmitoyl)-*sn*-glycero-3′phosphorylethylamide; PC: phosphatidylcholine; PS: phosphatidylserine; RES: reticuloendothelial system; IFN-γ: interferon-γ; AM: alveolar macrophage.

Introduction

Cancer metastasis

The ability of malignant neoplasms to produce metastasis in organs distant from the primary site is responsible for most deaths from cancer [1]. Metastases can be located in different organs and in different anatomical locations within the same organ. These aspects can exert a significant influence on the response of tumor cells to therapy [1]. Moreover, primary malignant neoplasms and metastases contain multiple cell populations exhibiting a wide range of biological heterogeneity in such parameters as cell surface properties, antigenicity, growth rate, karyotype, sensitivity to cytotoxic drugs, invasiveness, and the ability to metastasize [1–4]. The corollary of these findings is that the successful treatment of metastasis will require the development of new agents or therapeutic modalities that can circumvent both the existing cellular heterogeneity of neoplasms and the development of tumor cells resistant to therapy.

During the last few years, increasing evidence has shown that activated macrophages can meet these demanding criteria, and thus they offer an additional approach for treatment of disseminated cancer [5–7].

In vivo *targeting of immunomodulators*

Considerable attention has been focused on the use of synthetic phospholipid vesicles (liposomes) to selectively target therapeutic agents to different tissues [8–13]. Unfortunately, most attempts to target liposomes containing anticancer agents to primary solid neoplasms or, more important, to metastases, have failed because liposomes introduced into the blood stream cannot traverse capillaries to extravasate the circulation. Instead, like any other circulating foreign particle, liposomes are rapidly cleared by fixed and free phagocytic cells [5–7, 12, 14]. We have taken advantage of this fact to target liposomes containing immunomodulators to cells of the reticuloendothelial system (RES). Since macrophages are an important component of host defense against infections and cancer [6, 7], we have proposed that the systemic administration of liposomes could achieve selective delivery of agents to phagocytic cells and thus enhance host resistance to viral diseases [15], microbial diseases [14], and cancer metastasis [16, 17].

The activation of macrophages to the tumoricidal state

Although tumor cell populations are heterogeneous with regard to many characteristics, they appear to be susceptible to destruction by activated (tumoricidal) macrophages [6–8, 16, 17]. There are two major pathways to activate macrophages *in vivo*. Macrophages can become activated subsequent to interaction with microorganisms or their products, such as endotoxins and bacterial cell wall skeleton. Muramyl dipeptide (MDP), a defined component of the bacterial cell wall [18, 19], activates the immune system [20] without inducing the adverse side reactions such as allergic reactions and granuloma formation associated with the administration of microorganisms or their products [21, 22]. MDP is a water-soluble, low molecular weight (MW = 495), synthetic moiety of N-acetylmuramyl-L-alanyl-D-isoglutamine. Among many lipophilic MDP derivatives, N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1′,2′-dipalmitoyl)-*sn*-glycero-3′phosphorylethylamide (MTP-PE) is also available [23]. Although MDPs influence several macrophage functions *in vitro*, comparable effects have not been observed *in vivo* because the molecules are rapidly excreted in the urine after parenteral administration [20, 24, 25]. Even when injected at very high doses, MDPs fail to induce significant activation of macrophages and, hence, antitumor activity [20, 26, 27].

In vivo activation of macrophages can also be accomplished by interaction with lymphokines released by antigen- and mitogen-stimulated T lymphocytes. Lymphocytes interact in a highly specific fashion with target cells bearing appropriate receptors. In the case of macrophages, activation is produced by lymphokines generally referred to as macrophage-activating factors (MAF) [28–30], which include interferon gamma (IFN-γ) [31–33].

Activated macrophages can recognize and destroy neoplastic cells both *in vitro* and *in vivo* while leaving nonneoplastic cells unharmed. Data generated in rodent and human systems indicate that, at least *in vitro*, tumoricidal macrophages can discriminate between neoplastic and nonneoplastic cells by a process that is nonimmunologic in nature, independent of transplantation antigens, species-specific antigens, tumor-specific antigens, cell cycle time, or various phenotypes associated with transformation, and is dependent on cell-to-cell contact [7, 16, 17, 34–37]. Moreover, macrophage lysis of murine tumor cells appears to be nonselective for resistant tumor cell variants [38].

Because activated macrophages are able to kill phenotypically diverse tumor cells, including cells that are resistant to killing by other components of the host defense system

(T cells, NK cells) and various anticancer drugs [7, 8], and leave normal cells unharmed, they present an attractive possibility for the treatment of disseminated heterogeneous metastatic disease.

Interaction of macrophages with liposomes containing immunomodulators *in vitro*

Binding and phagocytosis of liposomes by macrophages

In order for liposomes to carry activating signals to macrophages, they must retain the entrapped compound until they bind to and become phagocytosed by cells of the RES. We and others have evaluated multilamellar vesicles (MLV) with different phospholipid compositions and have identified certain classes of phospholipids that are preferentially recognized by macrophages. Specifically, the inclusion of negatively charged phospholipids such as phosphatidylserine (PS) in MLV consisting of phosphatidylcholine (PC) enhances binding to and phagocytosis by macrophages, whereas uncharged MLVs composed exclusively of PC are not efficiently taken up by macrophages [39–50]. In fact, the inclusion of PS within PC MLV leads to recognition of these liposomes by every cell type of the RES. These include mouse peritoneal macrophages [45–50], mouse Kupffer cells [51–53], rodent alveolar macrophages [54, 55], human peripheral blood monocytes [29, 30, 43, 56–59], and human alveolar macrophages [60].

In vitro *activation of mouse macrophages by liposomes containing immunomodulators*

Immunomodulators entrapped within PC/PS liposomes can render cells of the monocyte-macrophage series tumoricidal much more effectively than the same concentrations of unencapsulated immunomodulators [31–33, 47, 48]. Efficient *in vitro* activation of macrophages has been achieved by employing liposomes containing entrapped MDP, MTP-PE, IFN-γ, or MAF [54, 55, 57–61]. Studies with liposomes containing MAF or IFN-γ clearly demonstrated the superiority of these vesicles in delivering signals to macrophages [31, 61]. These initial investigations on the activation of tumoricidal properties in rodent macrophages by liposome-encapsulated MAF or IFN-γ have been expanded to the human system with equivalent results. Liposome-encapsulated human MAF or IFN-γ were at least 800 times more efficient than free lymphokines in rendering human blood monocytes cytotoxic to allogeneic tumor cells *in vitro* [29, 30, 32, 33].

Liposomes as carriers for macrophage activators *in vivo*

In situ *activation of macrophages by liposomes containing immunomodulators*

The clearance of circulating liposomes by phagocytic cells of the RES as well as by free blood monocytes [12, 14, 39–45, 62] provides a preferential delivery system of immunomodulators because phagocytosed biologically active materials are released into the cytoplasm of the phagocyte. This targeting thereby avoids the problems of dilution, serum protein binding, and rapid clearance, and minimizes the elicitation of undesirable side effects [11–15]. The natural localization pattern allows efficient targeting of liposomes and their contents to various macrophage compartments in the body [6–8, 40].

The optimal type of liposome for delivering macrophage-activating agents to the lung to augment the tumoricidal activity of lung macrophages was determined. Much like the conditions required for optimum binding and activation of macrophages, large MLV (more than 0.1 μm in diameter) prepared from PC and PS (7 : 3 mol ratio) arrested most efficiently in the lung vasculature [39, 63].

Uptake of liposomes by blood monocytes was demonstrated by showing the intravenous injection of PC/PS MLV liposomes containing fluorescein-labeled bovine serum albumin resulted in localization of fluorescence within lung macrophages and alveolar macrophages (AM), which were recovered by pulmonary lavage [45]. Similarly, AM recovered after i.v. injection of PC/PS MLV containing immunomodulators exhibited tumoricidal activity [39, 42, 49]. In contrast, macrophages recovered from control mice injected with free immunomodulators or with liposomes containing placebo preparations were devoid of cytotoxic activity [39, 42, 49]. Neutral PC-MLV liposomes containing lymphokines, which show only very limited retention in the lung, were ineffective in activating AM *in situ*.

These data show that negatively charged MLV liposomes (PC/PS 7:3 mol ratio) localize efficiently in the lung and the macrophage-activating agents encapsulated within such liposomes can successfully activate blood monocytes *in situ* [39, 45].

These findings were extended by the observation that MAF and MDP delivered together in liposomes produced a synergistic effect [64]. Subthreshold amounts of MAF or MDP that singly did not have an effect on AM would, when injected together in MLVs, activate mouse AM to become tumoricidal. A further improvement in the use of encapsulated immunomodulators is afforded by lipophilic agents such as MTP-PE. Since low-molecular-weight solutes such as hydrophilic MDP can leak out of liposomes, we examined the possibility that MTP-PE inserted in the phospholipid bilayers of liposomes would be retained more efficiently within macrophages and thereby promote longer periods of tumoricidal activity [27, 42]. The liposomes containing the lipophilic MTP-PE were indeed superior to liposomes containing water-soluble MDP for activation of macrophages. This was demonstrated in two ways [42]. First, the i.v. injection of liposomes containing a dose of MTP-PE equal to MDP led to higher levels of AM-mediated cytotoxicity *in vitro*. Second, AM harvested from mice given i.v. injections of liposomes containing MTP-PE maintained tumoricidal activity for a longer period (5 days) than macrophages harvested from mice inoculated with liposome-encapsulated MDP (3 days).

It has been suggested that once macrophages phagocytose liposomes containing MDP, the liposomes function as a slow-release depot: encapsulated material is released from them over a sustained period of time [12, 39, 65]. The extent of release and the possible equilibration of MDP with the extracellular medium are determined by the integrity of liposome membranes. However, MTP-PE, which is only slightly soluble in water, would remain active for longer periods until it is degraded. Since liposomes can be seen inside macrophages for several days after phagocytosis [47, 48], the degradation of MTP-PE incorporated into the liposome phospholipid bilayer is apparently relatively slow and inefficient.

Duration of tumoricidal macrophage activity

Any potential therapeutic modality for metastatic disease must provide sustained activity. In order to assess whether AM activated by liposomes containing MDP maintain tumoricidal properties over an extended period, AM were harvested and assayed for their tumoricidal activity 1, 2, 3, or 4 days after the i.v. injection of a single dose of MLV (2.5 μmol) containing 10 μg or 100 μg of MDP, MTP-PE, or a combination of both [41]. On day 1, AM harvested from all the mice were cytotoxic against the syngeneic tumor. By day 3, however, AM harvested from mice that received MLV with hydrophilic MDP exhibited less than 20% cytotoxicity. In contrast, at any time point, AM harvested from mice that received MLV containing MTP-PE consistently exhibited significantly higher levels of tumoricidal ac-

tivity. The i.v. injection of MLV containing the combination of both MDP and MTP-PE did not significantly increase the level of AM-mediated cytotoxicity over that obtained with MLV containing MTP-PE alone [41]. Repeated i.v. injections with MLV containing MDP produced a continuous presence of activated AM, probably by activating fresh blood monocytes.

Macrophage activation is a T-lymphocyte-independent process

The immune system of a tumor-bearing animal may be suppressed. It is therefore imperative to determine if macrophage activation by liposomes containing immunomodulators is dependent on viable T-lymphocytes for release of lymphokines. To answer this question, we studied three groups of immunosuppressed mice: mice treated by ultraviolet radiation, thymectomized and whole-body X-irradiated mice, and athymic nude mice. All mice were injected i.v. with PC/PS MLV containing MTP-PE. AM recovered from these mice were found to be tumoricidal, whereas control empty liposomes had no effect [66]. These data show that the *in situ* activation of macrophages by liposomes containing immunomodulators is independent of T-lymphocytes [66].

Cyclosporine A is an inhibitor of T-lymphocyte-mediated immunity. Administration of cyclosporine A followed by i.v. injection of MTP-PE liposomes into mice rendered the AM from these mice significantly cytotoxic [67]. Cyclosporine A had no effect on the generation of tumoricidal macrophages, suggesting that the T-lymphocytes affected by cyclosporine A do not play a role in the activation of macrophages by liposomes containing immunomodulators. Furthermore, this study presents the possibility of macrophage therapy for transplant patients who are treated with cyclosporine A to prevent graft rejection [67].

Treatment of melanoma metastasis by intravenous injections by liposomes containing immunomodulators

Eradication of metastasis in a murine model

In the next set of studies we began to examine the possibility that systemic adminstration of immunomodulators encapsulated in liposomes could enhance host destruction of lung and lymph node metastases [41]. The B16-BL6 melanoma cell line, which is syngeneic to C57BL/6 mice, was used as the primary model to determine the effectiveness of liposome-encapsulated materials in the treatment of metastases. After implantation in the foot pad, this tumor metastasizes to lymph nodes and the lungs in more than 90% of mice [64]. Mice were injected with melanoma cells and 4–5 weeks later, when the tumors had reached a size of 10–12 mm, the leg bearing the tumor, including the popliteal lymph node, was amputated. Three days later, the mice were injected i.v. with PC/PC (7:3 mol ratio) MLV containing immunomodulators or a control preparation twice weekly for 4 weeks.

In this tumor model, spontaneous metastases in the lungs and lymph nodes were well established at the time liposome treatment began. Many individual metastases could be seen macroscopically. In different experiments, all mice treated intravenously with saline, with free MAF, with free MDP, or with liposomes containing saline were dead by day 90 of the experiment. In marked contrast, >66% of mice injected intravenously with liposome-encapsulated immunomodulators were cured of the disease. In this tumor system, we estimate that at the beginning of the liposome treatment, the metastases contained 10^7 cells. Since the median survival time of mice injected with as few as 10 viable B16 cells (admixed with 10^6 dead cells) is 40–50 days [28, 41, 68], the results suggest that the residual tumor burden in the liposome-treated surviving mice must

have been reduced to less than 10 viable cells [41, 68].

Since synergistic activation of the tumoricidal properties in AM by unencapsulated MAF and MDP has been previously shown to occur *in vitro* [11–13, 69, 70], we investigated whether the combination of MAF and MDP (within the same liposome) could produce synergistic activation of lung macrophages *in vivo* and enhanced destruction of lung melanoma metastases [64]. The i.v. injection of neither free MAF nor free MDP produced systemic activation of macrophages *in situ*. The i.v. admistration of MLV containing optimal doses of MAF or MDP generated tumoricidal properties in lung macrophages [64]. On the other hand, i.v. adminstration of MLV containing a 1/20 dilution of MAF or 1/20 dilution of MDP did not activate the lung macrophages to become tumor cytotoxic. However, when both these subthreshold doses of MAF and MDP were combined and encapsulated within the same MLV, significant *in situ* activation was produced.

Therapeutic experiments were carried out in mice with relatively large metastases. We postponed the start of treatment until 7 days after the resection of the primary neoplasms to allow examination of the hypothesis that MAF and MDP encapsulated within the same liposome would act synergistically to activate macrophages *in situ*, thus destroying more metastatic tumor cells. At the time of first i.v. treatment, spontaneous pulmonary metastases were well established, with some lung metastases of 1 mm in diameter. Liposomes were injected twice weekly for 4 weeks. Practically all the mice receiving saline alone or MLV containing diluted MAF or diluted MDP died by day 90 of the experiment. Multiple i.v. injections of liposomes containing either optimal MDP or optimal MAF doses resulted in long-term survival (longer than 250 days) of 27% of the animals (5 of 18). Mice treated with MLV containing both diluted MAF and diluted MDP had high survival rates, with 50% of the mice alive at day 250 [64].

The synergistic effects of MAF and MDP were recently surpassed with the use of MTP-PE and IFN-γ. MTP-PE was previously shown to be more efficient than MDP in its ability to render AM tumoricial [43]. This is due to the lipophilic nature of MTP-PE, which increases the active lifetime of this molecule. MTP-PE and IFN-γ were administered within the same PC/PS liposomes to mice that had been injected in the hind foot pad with B16-BL6 melanoma cells [71]. Mice were treated with liposomes either 3 or 10 days after amputation of the hind foot pad. In untreated animals, this led to the formation of small (< 0.1 mm) or large (0.1–1.0 mm) lung metastases, respectively. Treatment with liposomes containing MTP-PE and IFN-γ resulted in the long-term survival of 70% of the mice with small lung metastases and 50% of mice with large lung metastases. Control injections of liposomes containing saline resulted only in a 10% long-term survival rate [71]. Thus, the use of synthetic MTP-PE and IFN-γ in liposomes is efficacious in treating lung metastasis. Importantly, this combination of immunomodulators stimulates macrophages to a level of tumoricidal activity that is effective against large metastases. Since this is often the case at the time of diagnosis, MTP-PE and IFN-γ may have direct clinical applications.

The successful treatment of metastases by the intravenous injection of liposomes containing immunomodulators has also been effected in different tumor cell models, such as mouse fibrosarcomas [72–74] and melanomas [75], colon carcinomas [76], and autochthonous mouse tumors induced by chronic UV irradiation [77].

Treatment of canine osteosarcoma with MTP-PE liposomes

Canine osteosarcoma is a spontaneous malignancy that results in death from lung metas-

tasis. Amputation of the affected limb results in a median survival time of only 3–4 months. In a recent report [78] of the therapeutic efficacy of liposomal MTP-PE for metastasis, dogs with osteosarcoma underwent amputation of the affected limb, after which they were injected i.v. twice weekly for 8 weeks with PC/PS liposomes containing MTP-PE or saline. In this double blind study, the median survival of dogs treated with MTP-PE liposomes was 222 days. The median survival time of dogs treated with control saline liposomes was only 77 days. Furthermore, four dogs in the MTP-PE treatment group were alive and free of metastasis one year after surgery [78]. No toxic effects were reported. This preclinical study indicates that macrophage activation may be useful for treatment of metastasis.

Liposome therapy induces activation of in situ *macrophages*

The regression of established metastases after the systemic administration of liposomes containing MDP, MAF, or MTP-PE is attributed to the activation of macrophages to the tumoricidal state. Several lines of evidence support this conclusion. First, lung macrophages are not activated by macrophage-activating agents encapsulated within liposomes that are not retained in the lung [26]. Second, the pretreatment of tumor-bearing animals with agents that are toxic for macrophages (silica, carrageenan, hyperchlorinated drinking water) before systemic therapy with liposome-encapsulated MDP or MAF abrogates the response to liposome therapy, and such animals rapidly die of metastatic disease [26]. Third, the possible involvement of T-lymphocytes as effector cells is excluded by the finding that systemic activation of macrophages by liposome-encapsulated MDP can be accomplished in athymic nude mice and in adult thymectomized, X-irradiated mice. Fourth, i.v. injections of macrophages activated *in vitro* by incubation with liposome-encapsulated MDP produces a reduction in metastatic burden comparable to that achieved by systemic admistration of liposome-encapsulated activators [26]. Finally, direct evidence that the regression of established metastases was associated with tumoricidal macrophages comes from morphological and functional analysis of macrophages isolated from pulmonary metastases [79]. Immunofluorescence and electron microscopic analyses revealed that 24 hours after the tumor-bearing mice were given i.v. injections of liposomes, 15% of the AM and 5% of the metastasis-associated macrophages contained phagocytosed liposomes. However, only macrophages isolated from lungs or metastases of mice given injections of liposomes containing activators (treatment success) and not macrophages from mice treated with empty liposomes (treatment failure) were tumoricidal against target cells *in vitro* [79].

Conclusions

The uncontrolled growth of metastasis resistant to conventional therapies is a major cause of death from cancer. Multiple metastases can exhibit different sensitivities to therapeutic modalities, implying that to be successful, therapy of disseminated metastases must circumvent neoplastic heterogeneity and tumor cell resistance to therapy. Furthermore, this therapy must be selective in its actions and not adversely affect normal cells.

Appropriately activated macrophages can fulfill these demanding criteria. Macrophages can be activated to become tumoricidal by interaction with phospholipid vesicles (liposomes) containing various immunomodulators. Tumoricidal macrophages can recognize and destroy neoplastic cells *in vitro* and *in vivo*, while leaving nonneoplastic cells unharmed. Although the exact mechanisms by which macrophages discriminate between tu-

morigenic and normal cells is unknown, it is independent of such tumor cell characteristics as immunogenicity, metastatic potential, and sensitivity to cytotoxic drugs. Moreover, macrophage destruction of tumor cells apparently is not associated with the development of tumor cell resistance.

Intravenously administered liposomes are cleared from the circulation by phagocytic cells. The endocytosis of liposomes containing immunomodulators generates cytotoxic macrophages *in situ*. The multiple administrations of such liposomes have been shown to bring about eradication of cancer metastases in several tumor systems.

It is important to note that even the destruction of 99.0% of cells in a metastasis measuring 1 cm^3 would leave 10^6 cells to proliferate and kill the host. The ability of tumoricidal macrophages to distinguish neoplastic from bystander nonneoplastic cells presents an important new approach for the treatment of the few tumor cells that escape destruction by conventional therapeutics.

Acknowledgements

This work was supported in part by funds from NIH Training Grant T32 CA09598-01, by the Smith Education Fund, and by grant R35 CA42107 from the National Cancer Institute, National Institutes of Health.

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