Sustained Cytokine Delivery for Anticancer Vaccination: Liposomes as Alternative for Gene-transfected Tumor Cells

Frank J. Koppenhagen, Zaruhi Küpcü, Gerhard Wallner, Daan J. A. Crommelin, Ernst Wagner, Gert Storm, and Ralf Kircheis

Department of Pharmaceutics, Utrecht University, 3508 TB Utrecht, the Netherlands [F. J. K., D. J. A. C., G. S.]; and Bender & Co. GmbH, A-1121 Vienna, Austria [Z. K., G. W., E. W., R. K.]

ABSTRACT
Vaccination with tumor cells genetically engineered to produce interleukin (IL)-2 is an attractive strategy to enhance antitumor immune responses. The improved antitumor immunity upon vaccination with IL-2 gene-modified tumor cells may be due to the prolonged presence of the cytokine at the vaccination site. Because liposomes have been used for sustained delivery of a variety of agents, we compared the protective effect of vaccines consisting of IL-2 gene-modified B16 melanoma cells to that of vaccines composed of IL-2 liposomes and irradiated melanoma cells. The results indicate that both approaches equally protect against a lethal challenge with B16 melanoma cells. More than 20% of the protected animals developed vitiligo at the vaccination and/or tumor challenge site.

INTRODUCTION
Immunotherapy is under investigation for application in cancer treatment. One of the agents investigated extensively for application in immunotherapy of cancer is IL-2, a cytokine involved in the regulation of the immune response. Previous strategies for IL-2 immunotherapy relied on systemic cytokine administration to enhance antitumor immunity. However, this approach is often accompanied by severe toxicity (1, 2). Local cytokine administration directly into the tumor or peritumoral tissue can avoid these side effects and still result in systemic antitumor immunity (3, 4). Most biotherapies require, however, a prolonged presence of the cytokine, and this is difficult to achieve by local administration of recombinant cytokine proteins.

The usage of gene-modified tumor cells as the source of the cytokine (5–7) may offer considerable advantages over systemic or locoregional cytokine administration. First, it provides prolonged expression of the cytokine, and second, high cytokine levels may be obtained locally at the vaccination site, thus satisfying the paracrine nature of action of cytokines while avoiding systemic toxicity (8–10). On the basis of the success of preclinical studies in animals, several ex vivo gene therapy clinical trials have been initiated using IL-2, IL-4, IL-7, and GM-CSF gene-modified tumor cells (11–14). However, the genetic engineering approach faces a number of obstacles in clinical application, such as safety concerns and technical complexity. Particularly, the high variability between tumor cells of different patients in terms of transfection efficiency is a major problem because the vaccination efficiency was found to be dependent on the cytokine dose (9, 15, 16). Recently, the use of sustained cytokine release systems, such as microspheres or liposomes, has been proposed as a technically simpler approach in cancer vaccine design (17, 18). We were interested in directly comparing gene-modified tumor cells as a paracrine cytokine source with a vaccine composed of irradiated tumor cells and IL-2 liposomes for their efficiency to evoke protective immunity. Liposomes have been used as sustained release systems for a variety of molecules, including cytokines (18–21). Because large-scale production of liposomes is possible (22), cytokine-containing liposomes could be used as an off-the-shelf product to be admixed with irradiated tumor cells prior to vaccination. Here, we demonstrate that vaccines composed of irradiated melanoma cells admixed with liposomal rIL-2 are as effective as IL-2 gene-modified tumor cells and markedly more effective than irradiated cells alone in evoking protective immunity against a subsequent tumor challenge. Interestingly, a significant percentage of the protected animals developed vitiligo at the vaccination and/or tumor challenge site.

MATERIALS AND METHODS
Reagents. Egg l-a-phosphatidylcholine and egg l-a-phosphatidylglycerol were obtained from Lipoid KG (Ludwigshafen, Germany). Proleukin, human desalanyl 127-ser rIL-2 was obtained from Chiron (Amsterdam, the Netherlands). Acetonitrile was obtained from Biosolve (Barneveld, the Netherlands), and PFA was obtained from Sigma Chemical Co. (St. Louis, MO).

Liposome Preparation. Proleukin was reconstituted according to the manufacturer’s instructions and diluted to 100 μg/ml with water. Liposomes, consisting of egg l-a-phosphatidylcholine and egg l-a-phosphatidylglycerol in a molar ratio of 9:1, were prepared using the “film method.” The lipids were dissolved in methanol-chloroform (9:1, v/v) and evaporated to
IL-2 Liposomes for Anticancer Vaccination

**In Vitro Release of rIL-2 from Liposomes.** Release of rIL-2 from liposomes was assessed at 37°C in 10% sucrose containing 0.05% sodium azide and 0.5% human serum albumin. At various time points, samples were taken. Released rIL-2 was separated from the liposomes by ultracentrifugation (1 h at 250,000 × g). The amount of rIL-2 was determined by HPLC as described above.

**B16 Melanoma Cells.** B16F10 cells were transfected with the murine IL-2 gene as described previously by means of the Adenovirus-Enhanced Transferrin system (16, 24). The transfected cells were irradiated (50 Gy) to prevent proliferation of the cells, harvested, washed, counted, frozen in vials, and stored in liquid nitrogen. The amount of IL-2 produced by the cells was estimated by thawing one of the vials, seeding the cells in a cell culture flask, and growing the cells in DMEM supplemented with 10% FCS. At 1, 2, 3, and 7 days after transfection, the medium was replaced, and the amount of produced IL-2 in the cell medium was determined by a murine IL-2 ELISA (Becton Dickinson).

**Vaccination Protocol.** Animal experiments were performed in compliance with regulations for animal experiments (Amt der Wiener Landesregierung, Magistratsabteilung 58, application no. MA58-859/95). Female C57Bl/6 mice (Bomholtgaard, Denmark) were vaccinated twice on days 1 and 8 by s.c. injections in the back with 2 × 10^7 irradiated B16F10 cells in normal culture medium (RPMI 1640, 10% FCS, supplemented with 10% FCS). At 1, 2, 3, and 7 days after transfection, the medium was replaced, and the amount of produced IL-2 in the cell medium was determined by a murine IL-2 ELISA (Becton Dickinson).

**CTL Assay.** Splenocytes of C57Bl/6 mice immunized with irradiated B16F10 cells or irradiated B16F10 mixed with IL-2 liposomes (loaded with an IL-2 dose of 300 ng or of 2.3 μg) or of naive mice were isolated 12 days after the last vaccination. Splenocytes were restimulated for 5 days with PFA-fixed B16F10 cells in normal culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, and 1 μg/ml gentamicin) supplemented with 15 units/ml recombinant murine IL-2. Cytolytic activity was determined using a 51Cr-release assay against C57Bl/6 melanoma cells admixed with free or liposomal rIL-2. Two groups of animals were injected with irradiated IL-2 gene-transfected B16F10 cells expressing 350 or 700 ng of IL-2 per 2 × 10^7 cells in 24 h, respectively. Control animals received buffer only, irradiated tumor cells only, irradiated tumor cells admixed with empty liposomes, or irradiated tumor cells admixed with both empty liposomes and 700 ng of free rIL-2. A challenge with a tumorigenic dose of 1 × 10^7 B16F10 cells was given s.c. at the contralateral site on day 15, and tumor development was monitored until day 80 after tumor cell inoculation. The differences between the vaccination groups were statistically analyzed by the Mantel-Haenszel test and Fisher’s exact test, and the correlation coefficient r between the appearance of vitiligo and the protection against tumor challenge was calculated by Pearson’s correlation (using a GraphPad Prism 228 software).

**HPLC Determination of rIL-2 in Liposomes.** HPLC of rIL-2 in liposomes was performed after removal of the phospholipids via the Bligh and Dyer extraction method (23). Briefly, a 100-μl sample was admixed with 225 μl of methanol and 125 μl of chloroform. Phase separation was obtained by the addition of 125 μl of 0.1 m HCl and 125 μl of chloroform. After mixing and 5 min of centrifugation (Eppendorf 5414), 100 μl of the upper (water/methanol) layer were applied to a Phenomenex W-Porex C4 column. The mobile phase consisted of 52.5% (w/w) acetonitrile in water, containing 100 mM NaClO₄ and 10 mM HClO₄ (both from Merck), the flow rate was 1 ml/min. A 783A UV detector at 205 nm (Applied Biosystems, NJ) was used for detection.

**Determination of rIL-2 in Liposomes.** HPLC of rIL-2 in liposomes was performed after removal of the phospholipids via the Bligh and Dyer extraction method (23). The dispersion was freeze-dried overnight in 1-ml aliquots using a Christ Alpha 1-2 freeze-dryer. The freeze-dried rIL-2 in liposomes was performed after removal of the phospholipids via the Bligh and Dyer extraction method (23). After 30 min, the liposomes were diluted with a 10% sucrose solution and washed twice by ultracentrifugation at 200,000 × g. Liposomes floating on the top of the liquid were collected. After thawing one of the vials, seeding the cells in a cell culture flask, and growing the cells in DMEM supplemented with 10% FCS. At 1, 2, 3, and 7 days after transfection, the medium was replaced, and the amount of produced IL-2 in the cell medium was determined by a murine IL-2 ELISA (Becton Dickinson).

**CTL Assay.** Splenocytes of C57Bl/6 mice immunized with irradiated B16F10 cells or irradiated B16F10 mixed with IL-2 liposomes (loaded with an IL-2 dose of 300 ng or of 2.3 μg) or of naive mice were isolated 12 days after the last vaccination. Splenocytes were restimulated for 5 days with PFA-fixed B16F10 cells in normal culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, and 1 μg/ml gentamicin) supplemented with 15 units/ml recombinant murine IL-2. Cytolytic activity was determined using a 51Cr-release assay against C57Bl/6 melanoma cells admixed with free or liposomal rIL-2. Two groups of animals were injected with irradiated IL-2 gene-transfected B16F10 cells expressing 350 or 700 ng of IL-2 per 2 × 10^7 cells in 24 h, respectively. Control animals received buffer only, irradiated tumor cells only, irradiated tumor cells admixed with empty liposomes, or irradiated tumor cells admixed with both empty liposomes and 700 ng of free rIL-2. A challenge with a tumorigenic dose of 1 × 10^7 B16F10 cells was given s.c. at the contralateral site on day 15, and tumor development was monitored until day 80 after tumor cell inoculation. The differences between the vaccination groups were statistically analyzed by the Mantel-Haenszel test and Fisher’s exact test, and the correlation coefficient r between the appearance of vitiligo and the protection against tumor challenge was calculated by Pearson’s correlation (using a GraphPad Prism 228 software).
determined by using a europium (Eu³⁺) release assay, as described previously (16). The cytolytic activity was measured after a 2-h incubation period of the restimulated splenocytes (effectors) with viable Eu³⁺-labeled B16F10 cells (targets). Specific lysis was determined using the following formula: % specific lysis = (experimental Eu³⁺ release − spontaneous Eu³⁺ release)/(maximal Eu³⁺ release − spontaneous Eu³⁺ release). Spontaneous release was 10% of the maximum release induced by 1% Triton X-100.

RESULTS AND DISCUSSION

In a variety of animal models, vaccination with cytokine gene-modified tumor cells has been shown to elicit protection against a tumor challenge and even, in some cases, to cause regression of established tumors (5–10, 16, 25, 26). However, a major limitation in the translation of this strategy to large-scale human tumor vaccine therapy is the labor intensity and difficulty of establishing a primary culture of each patient’s tumor cells and transfecting them with the appropriate vectors. Moreover, the efficiency of transfection may vary significantly (>100-fold) between tumors of different individuals. However, it has been shown that, for efficient vaccination, the cytokine dose must be within a therapeutic dose window (9, 15, 16). Because liposomes have been shown to confer sustained release properties to drug formulations, liposome incorporation might provide a technically simpler strategy to achieve a standardized sustained presence of cytokines at the vaccination site than genetically engineered cells. Indeed, the effectiveness of IL-2 liposomes as adjuvants in vaccination has been shown (18).

In the experiments presented here, the antitumor effect induced by a vaccine formulation consisting of rIL-2 liposomes admixed with irradiated tumor cells was compared with that induced by IL-2 gene-transfected tumor cells in the murine B16 melanoma model. This tumor model was used because it represents a poorly immunogenic tumor in which vaccination with irradiated wild-type cells produces little protective immunity against challenge with live wild-type tumor cells. A comparison between gene-modified tumor cells and microparticles containing GM-CSF or IFN-γ was made previously in a similar vaccination model (17). However, because the cytokine dose seems to be most critical for the effect of IL-2 (in contrast to the less stringent dose dependency in the case of GM-CSF; Refs. 15 and 16), we tried to quantify our sustained release system concerning the amount of the entrapped cytokine, as well as the release kinetics.

To assure that both vaccine formulations are able to release IL-2 over a sustained period of time, in vitro experiments assessing the release of rIL-2 from liposomes and the production of IL-2 by gene-transfected tumor cells were performed. Fig. 1 shows that, indeed, both formulations are able to release IL-2 over a prolonged period of time in vitro. The IL-2 gene-transfected B16F10 tumor cells produce significant amounts of IL-2 over a period of 3 days. Some low IL-2 production is detectable up to 7 days. The release of rIL-2 from liposomes lasts for ~14 days. Previously, we showed that liposomes of this type remain present at the injection site for several days after s.c. injection (27). This, together with the slow-release properties, indicated that IL-2-containing liposomes in combination with irradiated melanoma cells may be used as an alternative to irradiated IL-2 gene-transfected melanoma cells for vaccination.

To assess the feasibility of this concept, we directly compared both approaches in one study. Fig. 2 demonstrates that rIL-2 liposomes admixed with irradiated tumor cells generated comparable systemic immunity against a challenge with viable melanoma cells when compared with IL-2 gene-transfected tumor cells. Highest efficacy was observed at doses of 60–300 ng of rIL-2 per immunization. Vaccination using lower (12 ng) or higher (1.5 or 2.3 µg) IL-2 doses resulted in a lower protection.

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* E. Wagner, unpublished data.
These dose-response correlations are very similar to the bell-shaped dose-response correlations shown for IL-2 gene-modified tumor cells in the same model as well as in other tumor models (15, 16). The observed dose-response correlations for systemic protection against tumor challenge were found to correlate with the CTL activity (Fig. 3). Immunization with irradiated cells admixed with IL-2 liposomes at an optimal IL-2 dose (300 ng) induced a moderate but significant increase in the CTL activity against B16F10 tumor cells. This effect was reduced when too-high IL-2 doses (2.3 μg) were used. To induce systemic immunity, either gene modification or liposomal incorporation of rIL-2 was found to be essential because animals immunized with irradiated tumor cells admixed with free rIL-2 failed to generate significant systemic protective responses at any dose tested. Also, irradiated tumor cells supplemented with free rIL-2 and empty liposomes did not elicit a significant protective effect. All control animals (no immunization, immunization with irradiated tumor cells, or immunization with irradiated tumor cells and empty liposomes) developed a lethal tumor. Because the use of free rIL-2 as supplement for the irradiated tumor cell vaccine was not effective, the observed protective immunity upon vaccination with IL-2 gene-transfected tumor cells or with IL-2 liposomes as supplement should be related to the prolonged presence of IL-2 at the vaccination site, providing additional support for the hypothesis that some level of sustained presence of the cytokine at the site of antigen presence is important for achieving systemic protection (8-10). Because the therapeutic dose range does not seem to differ significantly between different tumor models or animal strains (9, 15, 16), one optimized sustained release formulation might be applicable to a broad variety of patients.

Remarkably, some of the protected animals showed vitiligo at the vaccination and/or the tumor challenge site (Fig. 4A). This effect was not seen in any of the control groups. Although, in this particular experiment, no vitiligo was observed with IL-2 gene-modified vaccines (because of the small number of animals), we have observed vitiligo in animals immunized with IL-2 gene-modified cells when looking at a larger number of experiments (Fig. 4B). Table 1 shows a summary of experiments where IL-2 gene-modified cells and/or IL-2 liposomes admixed with irradiated cells were used for vaccination against a challenge with viable B16 melanoma cells. Vitiligo was observed in 6 of 65 (9.2%) and 4 of 38 (10.5%) mice immunized with IL-2 gene-modified cells or IL-2 liposomes admixed with irradiated cells, respectively, both at optimal dose range (60–400 ng of
IL-2). There was only one single mouse with vitiligo found after immunization with irradiated cells (1.1%), and vitiligo has never been observed in nonimunized mice challenged with B16 cells. Noteworthy, all mice developing vitiligo were also protected from tumor challenge. Statistical analysis revealed a very high correlation coefficient $r = 0.9944$ ($P < 0.01$) between the appearance of vitiligo and the protection against tumor challenge. In the IL-2-vaccinated groups (in both gene-modified and IL-2 liposomes), vitiligo was observed in >20% of the protected animals. There were no significant differences between the IL-2 gene-modified vaccines and the IL-2 liposome vaccines, either in the appearance of vitiligo or in the degree of protection. The observed correlation between vitiligo and protection from tumor challenge with murine melanoma compares well with clinical data reported by Rosenberg and White (28), who showed the appearance of vitiligo in 26% of patients with metastatic melanoma who developed an objective response to high-dose IL-2-based immunotherapy, whereas there was no vitiligo seen in melanoma patients who did not respond to immunotherapy. There was also no vitiligo observed in patients with metastatic renal cancer treated with high-dose IL-2, irrespective of whether they were responders or nonresponders (28).

Although the correlation between occurrence of vitiligo and clinical outcome is still controversial (29), here, vitiligo was observed only in animals developing a protective antitumor response. It is hypothesized that the occurrence of vitiligo is due to an immune response directed against shared antigen(s) on melanocytes and melanoma tumor cells, resulting in killing of either cell type. Interestingly, the strict localization of vitiligo to vaccination and/or tumor challenge site. This might indicate that, in addition to shared antigens, a second stimulus is necessary to initiate an effective immune response. At the vaccination site, the sustained release of the IL-2 might be this second stimulus. At the tumor challenge site, additional stimuli may result from changes in the microvascular system and microinflammatory reactions accompanying the outgrowth of the tumor and the establishment of a tumor-induced microvascular bed.

In conclusion, in the B16 melanoma model, IL-2 liposomes admixed with tumor cells can be as effective as IL-2-gene transfected tumor cells in evoking a systemic immune response against a lethal tumor challenge. Because the slow-release characteristics of liposomes can be tailored and industrial-scale production of liposomes is feasible, they offer an attractive alternative to cytokine-gene transfection of tumor cells for therapeutic vaccination protocols.

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REFERENCES


Sustained cytokine delivery for anticancer vaccination: liposomes as alternative for gene-transfected tumor cells.


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