Regression of High-Grade Malignancy in Mice by Bleomycin and Interleukin-12 Electrochemogenetherapy

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Abstract

Purpose: Bleomycin electrochemotherapy has been successfully used in preclinical studies and clinical trials for treating squamous cell carcinoma (SCC) and adenocarcinoma; however, it is not effective for treating recurrent tumors or metastatic tumors, or for preventing tumor redevelopment. In this study, we explore the coadministration of bleomycin and interleukin-12 (IL-12) followed by electroporation for treating primary and metastatic tumors.

Experimental Design: Bleomycin, IL-12 plasmid DNA, or a combination of both were injected into high-grade malignant mammary tumors and SCCVIIfollowed by electroporation. The tumor growth, survival, metastasis in lungs, CTL activity, and vascular density were analyzed. The results were analyzed by the two-sided Student’s t test and Gehan’s Wilcoxon test.

Results: Coadministration of bleomycin and IL-12 via electroporation eradicates preestablished 4T1 mammary tumors in up to 60% of mice, inhibits metastatic tumor development, and extends the long-term survival. Likewise, coadministration of bleomycin and IL-12 via electroporation eradicates squamous cell carcinoma (SCC VII) in 100% of mice and prevents tumor redevelopment in 80% of mice. Neither bleomycin nor IL-12 alone is able to achieve the same therapeutic potency. The primary role of bleomycin is to inhibit the tumor vessel development; the primary role of IL-12 is to increase the immune response that extends the survival of treated mice and inhibits the tumor redevelopment.

Conclusions: This combination modality has great potential to be translated in a clinical setting for treating high-grade malignancies and for preventing tumor redevelopment.

Breast cancer is the second leading cause of cancer deaths in women today and is the most common cancer among women, excluding nonmelanoma skin cancers (The American Cancer Society, 2005). According to WHO, >1.2 million people will be diagnosed with breast cancer this year worldwide. Despite the improvement in early diagnosis and treatment strategies, novel and effective alternatives for treatment, such as electrochemogenetherapy, have not been explored in breast cancer.

SCC of the head and neck is the fourth most common malignancy among males. More than 40,000 cases are diagnosed per year in the United States, 60,000 in Europe, and 500,000 worldwide (1, 2). Patients with SCC of the head and neck are afflicted with a disease that profoundly influences the quality of life (3). Surgical treatment may affect essential functions, including breathing, eating, and communication.

Many patients are plagued with tumor redevelopment after surgery that proves fatal. Clearly, patients need an effective but less debilitating local control treatment.

Electroporation has been used extensively to deliver drugs and genes into cells. The first report of an in vivo application of electric field pulses in combination with chemotherapeutic drugs was published by Okino and Mohri (4). The process of injecting nondiffusing drugs, such as bleomycin, followed by electric pulses to transiently make the cell membrane permeable, allowing drugs to enter the interior of the cell, is called electrochemotherapy. Electroporation has shown to be efficient and safe, regardless of the route of administration. Although electrochemotherapy with bleomycin leads in many cases to complete regression of tumors, the tumors may redevelop after the treatment is finished. Therefore, it is important to explore therapeutic approaches that can prevent tumor recurrence.

Interleukin-12 (IL-12) is a proinflammatory cytokine with antitumor activity. The major functions of IL-12 are the...
stimulation and activation of CTLs and natural killer cells and induction of other cytokines, such as IFN-γ, tumor necrosis factor-α, and granulocyte macrophage colony-stimulating factor (17, 18). Clinical trials using either i.t. or s.c. delivery of IL-12 protein showed significant antitumor activity and CD8+ T-cell stimulation (19–21). In vivo electroporation of the IL-12 gene has induced tumor regression and induction of antitumor immune memory in several tumor models (22–26), but multiple administrations are required to eradicate tumors with diameters that are ≤5 mm. Using a bleomycin and IL-12 combination, Kishida et al. showed the induction of antitumor immune memory in 38% of mice using a melanoma model.

In this study, we explore bleomycin and IL-12 electrochemogenetherapy for simultaneously inhibiting 4T1 primary tumors and metastatic tumors in BALB/c mice and for treating large volume of SCCVII in C3H mice. 4T1 s.c. transplant tumors in BALB/c mice induce spontaneous metastatic tumors that can metastasize to the lung, liver, lymph nodes, and brain (27). This is an animal model for high-grade stage IV human breast cancer (28, 29). SCCVII is originated from the oral cavity of C3H mice and has similar characteristics to SCC of head and neck in humans (30). For the first time that we know of, it is shown that coadministration of IL-12 and bleomycin induces a complete regression of the high-grade breast tumors and inhibition of metastatic tumor development in a maximum 60% of mice. The same treatment eradicates tumors with diameters between 6 and 7 mm in 100% of mice and prevents tumor redevelopment in 80% of the tumor-eradicated mice. Different from Kishida et al.’s data (31), our results suggest that the function of IL-12 is to extend the survival of treated mice and to prevent the tumor redevelopment, whereas the role of bleomycin is to eradicate primary tumors and to eliminate metastatic tumors.

Materials and Methods

Tumor model, DNA delivery, IL-12 construct, and cytokine expression. 4T1 cells were provided by Kanomas Cancer Center (Detroit, MI), and SCCVII cells were obtained from Dr. Bert O’Mally (Maryland, WA). Cells were maintained in DMEM containing 10% fetal bovine serum. 4T1 tumors were generated by inoculating BALB/c mice s.c. with 1 × 10^6 4T1 cells in a 30-μL volume, and SCCVII tumors were generated by inoculating C3H mice with 2 × 10^3 cells in the same volume as used for 4T1 cells. We purchased 6-week-old female BALB/c mice, weighing 18 to 20 g, from LSU Animal Facility and maintained them under NIH guidelines approved by the Institutional Animal Care and Use Committee. Tumor growth was measured with a caliper, and tumor volume was calculated with the following formula: \( V = \pi/6(ab^2) \), where \( V \) = tumor volume, \( a \) = maximum tumor diameter, and \( b \) = diameter at 90 degrees to \( a \) (32). Injection (i.t.) of plasmid DNA, bleomycin, or both followed by electroporation, as well as determination of tumor regression and survival, were done according to protocol described previously (26). In each experiment, five animals for each treatment or control group were used to study tumor regression or survival. At least two independent experiments were done to confirm the result. Optimal electroporation variables for i.t. injection, optimized previously, were used as described before (26). The variables used for i.t. electroporation for i.t. injection, optimized previously, were used as described before (26).

Plasmid DNA of the IL-12 construct was obtained from Valentis, Inc. (Burlingame, CA) and contains DNA fragments encoding both p35 and p40 subunits in the same backbone, as described in a previous publication (33), and the two subunits are driven by two independent cytomegalovirus promoters and terminated by two independent bovine growth hormone polyadenylation signals (33). The control plasmid DNA consists of a deletion of the IL-12 gene from the IL-12 construct. The plasmid DNA was manufactured using the Qiagen Endo-Free Prep kit (Valencia, CA). Quality control of plasmid DNA, such as restriction enzyme digestion analysis and gel electrophoresis analysis, was done before use. Bleomycin (30 units per bottle) was purchased from GensiaSicor Pharmaceuticals (Irvine, CA).

To determine the expression of IL-12 and IFN-γ, we used the same protocols as were previously used to prepare samples and perform ELISA (26).

**Metastatic tumor analysis.** One week after the second administration of control plasmid DNA, bleomycin, IL-12 plasmid DNA, or both IL-12 and bleomycin, mice were euthanized, and the lungs were filled with Bouin’s fixative (Lab Chem, Inc., Pittsburgh, PA). The number of metastatic foci in the lungs of mice from each group was counted using dissection microscope. The picture was taken using a Nikon 995 digital camera.

**Fluorescent microscope-based CTL assay.** CTL activity was evaluated using a CyToxiLux kit (OncoImmunin, Inc., Gaithersburg, MD), a single cell-based fluorogenic cytotoxicity assay (34). Splenocytes were obtained 3 weeks after the treatment from 4T1 tumor-bearing mice. The effector cells from splenocytes were primed by coculture with mitomycin C–treated 4T1 tumor cells in a ratio of 25:1 for 3 days, in RPMI 1640 with 20% FCS. Effector cells were incubated with red fluorescence–labeled target 4T1 cells in a ratio of 100:1 in a volume of 200 μL for 3 hours and then incubated with the quenched green fluorescence–labeled caspase substrate for 1 hour. The apoptotic target cells (red/yellow color cells) were examined using an Olympus BX41 fluorescence microscope (Olympus, Melville, NY). CTL activity was calculated using the following equation: \( \% \text{ specific killing} = 100 \times \frac{\text{spontaneous apoptotic target cells} - \text{apoptotic target cells}}{\text{spontaneous apoptotic target cells}} \)

**Immunostaining analysis.** We did immunostaining to determine vessel density. The vessels in the tumor tissues were stained using an antibody to CD31, an endothelial cell marker (1:200), as was described previously in detail (26). The number of vessels was scored from a minimum of four microscopic fields from five independent tumors. The average number of vessels per field was determined under a microscope at ×40 magnification.

**Statistical analysis.** A two-sided Student’s t test was done to compare tumor growth, the number of vessels, CTL activity, and the expression levels of cytokines between treatment groups. The survival analysis was done using \( x^2 \) test followed by Gehan’s Wilcoxon’s test to compare means of individual treatments. \( P < 0.05 \) were considered statistically significant.

**Results**

The level of IL-12 expression and IFN-γ induction in tumors by coadministration of bleomycin and IL-12. Bleomycin induces single-strand and double-strand breaks in both isolated and intracellular DNA (35–37). Uptake of bleomycin via electroporation also induces cell death, including some of the cells that simultaneously uptake both bleomycin and IL-12. Therefore, the coadministration of bleomycin and IL-12 may reduce the level of IL-12 expression and inhibit the level of IFN-γ induction because induction of IFN-γ is dependent on IL-12 expression. To determine whether this is the case, the expression of IL-12 and IFN-γ was compared between tumors that received both bleomycin and IL-12, or IL-12 alone. Coadministration of bleomycin and IL-12 did not inhibit the level of IL-12 expression but reduced the level of IFN-γ induction (Fig. 1), suggesting that bleomycin does not abrogate but reduces IL-12-mediated Th1 type response. However, the inhibition of IFN-γ expression by coadministration of...
bleomycin and IL-12 is not significant compared with administration IL-12 alone ($P = 0.28$), indicating that the inhibition of Th1 response by coadministration is not a major concern.

**Inhibition of tumor growth and increase of survival time in mice treated with bleomycin + IL-12 electrochemogenetherapy.** Bleomycin electrochemotherapy showed a significant tumor regression of SCC of the head and neck (http://www.gene-tronics.com), and IL-12 electrogenetherapy induced antitumor memory in both melanoma (25) and SCC tumors in mice (26), but it is unknown whether the combination of bleomycin and IL-12 via electroporation delivery will be able to cure high-grade breast malignancy. To show this, a 4T1 breast tumor model was used because this tumor represents a high-grade malignant breast tumor, and s.c. transplanted tumors induce an aggressive metastatic tumor development in other organs. IL-12 plasmid DNA, bleomycin, and the combination of IL-12 and bleomycin were injected into the 4T1 tumors bearing on BALB/c mice via electroporation to evaluate their individual antitumor effects. Bleomycin + control DNA was not included in this experiment because our preliminary study did not show any observable difference between bleomycin alone and bleomycin + control DNA. As expected, neither bleomycin nor IL-12 alone were able to eradic the high-grade malignant 4T1 tumor growth, but the combination bleomycin and IL-12 electrochemogenetherapy was able to eradic tumors in 40% of mice and significantly extended the survival duration in mice compared with either IL-12 alone ($P = 0.009$) or bleomycin alone ($P = 0.013$; Fig. 2).

To further improve the therapeutic efficacy, an increased number of administrations were done. Two administrations of bleomycin + IL-12 plasmid DNA followed by electroporation induced complete tumor eradication in 60% of mice (Fig. 3). These results strongly suggest that the coadministration of bleomycin and IL-12 is more effective than either molecule alone for inhibiting 4T1 tumor growth and prolonging survival in mice ($P = 0.008$ and $P = 0.009$ for bleomycin and IL-12, respectively). These results also suggest that both bleomycin and IL-12 are required for treating high-grade breast malignancy.

The treated mice died from either primary tumor volume–dependent or tumor volume–independent causes. Primary tumor volume–dependent death is defined as death occurring when tumors reach 1.5 cm in diameter, when mice are euthanized for humane reasons. Tumor volume–independent death describes the natural death occurring before the primary tumor size reaches 1.5 cm in diameter. This type of death is tumor volume independent because it occurs before tumor diameter reaching to 1.5 cm and is assumed to be bleomycin treatment related because the death rate increased proportionally with the number of bleomycin administrations (Fig. 3C). On the other hand, IL-12 alone did not yield any tumor load–independent death, and all the mice were euthanized when tumors reached 1.5 cm in diameter. Likewise, coadministration of bleomycin with IL-12 reduced this type of death in mice (Fig. 3C).

The tumor volume–independent death was not likely due to the induction of cystic fibrosis by bleomycin, because BALB/c mice are resistant to bleomycin-mediated induction of cystic fibrosis (38). To determine the possible cause, different organs were removed and examined. It seems that bleomycin greatly reduced the volume of spleens, but coadministration of bleomycin with IL-12 reversed such side effects (Fig. 3D). The shrinkage of spleens was correlated with bleomycin treatment–associated death (Fig. 3D).

**Reduction of metastatic tumors in mice using bleomycin + IL-12 electrochemogenetherapy.** To further show the potential of this therapeutic approach for treating high-grade tumors, we determined the inhibition of spontaneous metastatic tumors by treating primary tumors with the combination of bleomycin and IL-12 electrochemogenetherapy. Mice treated with bleomycin plus IL-12 had the lowest number of metastatic tumors in lungs, and this number was significantly different from control (CTRL, $P = 0.0068$) and IL-12 groups ($P = 0.025$; Fig. 4). Surprisingly, mice treated with bleomycin alone also showed a very low number of metastatic tumors, whereas mice treated with IL-12 alone only yielded an insignificant reduction of metastatic tumors compared with the control group. Together, the results suggest that bleomycin plays a primary role for inhibition of metastatic tumor growth, and inclusion of IL-12 enhances the inhibition of metastatic tumor growth (Fig. 4).

**The cellular mechanism that is associated with therapeutic efficacy and mice survival.** To explore the cellular mechanism that accounted for the tumor eradication, inhibition of tumor metastasis, and extension of survival in mice, both tumor vessel density and CTL activity were determined from mice that received different treatments. Vessel density was determined because previous studies have shown that bleomycin inhibits tumor angiogenesis (39, 40). As expected, both bleomycin alone and the combination bleomycin + IL-12 treatments induced a significant inhibition of angiogenesis in tumors ($P = 0.00054$ for bleomycin and $P = 0.0012$ for bleomycin + IL-12; Fig. 5A). Tumors from mice treated with IL-12 showed a slight reduction in tumor vessel density compared with the control group (Fig. 5A). Interestingly, the magnitude of tumor vessel reduction in primary tumors was associated with the level of metastatic tumor inhibition by different treatments (Fig. 4E and Fig. 5A), suggesting that inhibition of primary tumor angiogenesis is a positive prognosis factor for the inhibition of tumor metastasis.
To explore the cellular immune response that was associated with the antitumor effect by the bleomycin + IL-12 electrochemogenetherapy, CTL activities were determined. A strong CTL activity was observed in mice treated with bleomycin + IL-12 electrochemogenetherapy compared with bleomycin alone (P = 0.000163; Fig. 5B). IL-12 alone also induced a relatively higher level of CTL activity compared with bleomycin but resulted in a lower level of inhibition of metastatic tumors, suggesting that bleomycin plays the primary role, but IL-12-mediated antitumor immune response plays a secondary role in inhibition of metastatic tumor growth. To further show the benefit of IL-12-mediated antitumor immune response by coadministration with bleomycin, we s.c. inoculated 4T1 tumor cells into wild-type BALB/c mice and the tumor-eradicated mice by bleomycin + IL-12 treatment and compared the difference in tumor growth rate. The tumor-eradicated mice that were inoculated with 4T1 cells are referred to as rechallenged mice. Tumors developed in both wild-type and the rechallenged mice, but a rapid tumor development (5-fold increase in tumor volume within 6 days) was detected in wild-type mice, and only a small tumor volume increase was found in the rechallenged mice (Fig. 5C). The tumor volume of rechallenged mice was only one of seven of the tumor volume of wild-type mice 2 weeks after the inoculation (Fig. 5C), showing the presence of antitumor immune memory in the rechallenged mice that may inhibit the tumor development.

Fig. 2. Single coadministration of bleomycin (BLM) + IL-12 inhibits tumor growth and prolongs survival of mice. See Fig. 1legend for abbreviation and electroporation condition; 0.5 unit of BLM, 30 μg of plasmid DNA encoding IL-12, or a combination of both were injected when tumors reached 4 to 5 mm in diameter followed by electroporation. Five mice were used for each treatment group, and two independent experiments were done. Days of treatment (arrows). A, tumor growth. B, survival curve.

Fig. 3. Two coadministrations of bleomycin (BLM) + IL-12 inhibit tumor growth and prolong survival of mice. See Figs. 1 and 2 legends for detail. Days of treatment (arrows). Five mice were used for each treatment group, and two independent experiments were done. A, tumor growth. B, survival curve. The survival was based on the primary tumor diameter (>1.5 cm) and natural death. C, primary tumor load—independent death. D, effect of bleomycin on spleen shrinkage that was associated with tumor volume—independent death. Spleens were collected from euthanized mice 10 days after the second administration.
Eradication of large volume SCCVII tumors and prevention of tumor redevelopment by bleomycin + IL-12 electrochemotherapy. The successful treatment of high malignant 4T1 tumors leads us to determine whether coadministration of bleomycin with IL-12 also provides benefit in treating other type of tumors. To determine this, SCCVII tumor model was used because successful clinical data was obtained by bleomycin electrochemotherapy for regressing SCC (http://www.genetronics.com). We are interested in knowing whether coadministration of bleomycin and IL-12 provides any further benefit. As expected, bleomycin alone eradicated tumors with a relatively large initial volume (100-180 mm³), but tumors redeveloped from the tumor-eradicated mice in 2 weeks (Fig. 6). Significantly, coadministration of bleomycin + IL-12 not only eradicated tumors but also prevented the tumor redevelopment from 80% of mice. The result indicates that coadministration of bleomycin and IL-12 is also beneficial for the tumor type that can be treated very effectively by bleomycin alone (Fig. 6). Importantly, tumor-free mice from the bleomycin + IL-12 treatment rejected the rechallenged SCCVII tumor cells from 40% of mice, suggesting presence of an antitumor immune memory.

**Discussion**

There are no conventional treatments that are effective for regressing recurrent tumors, high malignant tumors, and metastatic tumors. The novel therapies, bleomycin electrochemotherapy and IL-12 electrogenetherapy, have shown antitumor effect in a variety of tumors (6, 15, 16) and in the induction of antitumor immune memory (23, 25–27). The challenge is that IL-12 electrogenetherapy cannot eradicate either large volume tumors or high-grade malignant tumors (Figs. 2-3). Bleomycin electrochemotherapy alone can eradicate some large volume tumors (13, 14) but fails to eradicate high malignant breast tumors (Figs. 2-3) and melanoma (41). The question is whether a combination of both bleomycin and IL-12 via electroporation delivery can overcome the failings of either treatment alone, resulting in the eradication of high-grade malignant tumors and prevention of tumor redevelopment. Results clearly show that such a combination therapy is able to eradicate high malignant 4T1 tumors and large volume SCCVII tumors and prevent tumor redevelopment. Results from this work and Kishida et al.'s study independently show that the combination therapy is superior to either modality alone for tumor eradication and metastatic tumor growth inhibition in both a high-grade malignant breast tumor (Figs. 2, 3, and 6) and melanoma model (41).

How does the coadministration of bleomycin and IL-12 induce such a superior effect in tumor eradication and inhibition of metastasis compared with either alone? Our results (Figs. 2-4) are entirely different from Kishida et al.'s result (31). Using a high-grade breast cancer model, our data show that IL-12 and bleomycin play distinctive roles for regressing primary tumors, inhibiting metastatic tumors, and extending the survival time. Bleomycin is the primary factor that inhibits both the metastatic and primary tumor growth (Figs. 2-4), whereas IL-12 is the primary factor that extends the survival of mice by reducing tumor load–independent death (Fig. 3C). Increased tumor volume–independent survival by IL-12 is associated with induction of tumor-specific CTL activity (Fig. 3C versus Fig. 5B), suggesting that the activation of the
immune system may contribute to the survival of mice. Why does IL-12 treatment improve the survival of tumor volume–dependent mice but does not greatly improve the survival of tumor volume–dependent mice compared with bleomycin treatment (Fig. 2B and Fig. 3B)? The reason is that IL-12 is not very effective in inhibiting tumor growth but is very effective in improving the health of immune system in mice. Contrastingly, bleomycin-treated mice were not healthy and died regardless of the significant inhibition of primary tumor growth (Fig. 3A). Therefore, the benefit of coadministration of IL-12 is not the prevention of bleomycin treatment–associated death. Different from our finding, Kishida et al.’s results indicated that IL-12 but not bleomycin plays a key role in inhibiting metastatic tumor growth (41). The difference in roles of IL-12 and bleomycin as discovered from two studies may be due to the difference in metastatic tumor formation. We used a spontaneous metastatic tumor model, and Kishida et al. used a metastatic tumor model by pulmonary injection of tumor cells. Regardless, one agreeable result between these two studies is that both IL-12 and bleomycin are required for complete regression of tumors and are beneficial for inhibiting metastatic tumor growth (Figs. 3 and 4; ref. 41). This result may not be IL-12 specific because coadministration of IL-2 with bleomycin is also beneficial for regressing tumors, but addition of IL-2 failed to prevent tumor redevelopment in some of the tumor eradicated mice (42).

Another benefit of coadministration of bleomycin and IL-12 via electroporation is the prevention of tumor recurrence. Some of the bleomycin-treated mice showed transient tumor regression or tumor growth arrest for 30 days for 4T1 tumors after tumor inoculation; however, the tumors started to grow very aggressively after 30 days from both tumor-eradicated and arrested mice. All the mice died or had to be sacrificed by day 55 for 4T1 tumors (Figs. 2 and 3). A similar observation was also found for SCCVII tumors, in which bleomycin eradicated large volume tumors, but tumors redeveloped in these mice (Fig. 6). Only combined bleomycin and IL-12 electrochemogenetherapy prevented the tumor recurrence once tumors were eradicated (Figs. 2, 3, and 6), showing the presence of antitumor immune memory by coadministration of bleomycin with IL-12. The induction of antitumor immune memory by coadministration of bleomycin with IL-12 was also supported by the inhibition of tumor development from rechallenged mice (Fig. 5C). The lack of complete rejection of rechallenged tumors is possibly due to the high malignancy of 4T1 tumor cells and is also possibly due to the relatively weak antitumor immune memory. To completely reject the rechallenged tumors, inclusion of other immune costimulatory molecules with IL-12 together may be necessary, which will be explored in the future.

In conclusion, this is the first study showing that bleomycin and IL-12 electrochemogenetherapy prevents tumor redevelopment and reduces bleomycin alone–associated tumor volume–independent death. The results from this and the other studies suggest that simultaneous coadministration of bleomycin + IL-12 is critical for eradicating aggressive tumors and inhibiting metastatic tumors.

Acknowledgments

We thank Drs. Changxia Xie and Shiguo Zu for assistance in preparing tumor cells for inoculation and removal of lungs from mice.

References


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*Clin Cancer Res* 2006;12:257-263.

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