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Award Number: DAMD17-03-1-0768

TITLE: Effect of Depleting Tumor-Associated Macrophages on Breast Cancer Growth and Response to Chemotherapy

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REPORT DATE: October 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Annual (15 Sep 2003 - 14 Sep 2004)

4. TITLE AND SUBTITLE
Effect of Depleting Tumor-Associated Macrophages on Breast Cancer Growth and Response to Chemotherapy

5. FUNDING NUMBERS
DAMD17-03-1-0768

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Tumor-associated macrophages (TAM) may comprise up to 50% of the tumor mass in breast cancer and are capable of producing estrogen and angiogenic cytokines that regulate the growth and angiogenesis of breast cancer. The goal of this project is to determine whether depletion of TAM, achieved by intra-tumoral injection of liposome-encapsulated dichloromethylene diphosphonate (clodronate), can slow tumor growth and improve the outcome of breast cancer treatment. Preliminary studies so far have revealed that daily intra-tumoral injection of liposome-encapsulated clodronate had no effect on the growth of subcutaneous breast cancer (4T1) model in mice. Whether liposome-encapsulated clodronate depletes tumor-assocaited macrophages in this model is currently under investigation.

14. SUBJECT TERMS
Macrophages, Breast Cancer, Chemotherapy

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

Tumor-associated macrophages (TAM) are one major component of tumor stroma. In breast cancer, TAM may comprise up to 50% of the tumor mass (1) and are capable of producing estrogen and angiogenic cytokines such as vascular endothelial growth factor and tumor necrosis factor alpha, that regulate the growth and angiogenesis of breast cancer (1-3). There is a positive correlation between high vascular grade and increased macrophage index, and a strong relationship between increased macrophage counts and reduced relapse-free survival as well as reduced overall survival in patients with breast cancers (3). In addition, the accumulation of TAM with thymidine phosphorylase is a potent prognostic indicator of early relapse in primary breast cancer (4). Despite the potential importance of TAM, current treatments of breast cancer have been primarily directed at breast cancer cells. Depletion of TAM may significantly improve the outcome of breast cancer therapy. The purpose of this study is to investigate weather depletion of TAM can slow breast tumor growth and improve the outcome of breast cancer treatment. Depletion of breast cancer TAM will be achieved using intra-tumoral injection of liposome-encapsulated dichloromethylene diphosphonate (clodronate), a specific and potent macrophage-depleting agent (5-8). Using a well established murine 4T1 breast cancer model (9), our specific objectives are: 1) To determine whether intra-tumoral injection of liposome-encapsulated clodronate can deplete TAM, 2) To determine the effect of depleting TAM on the tumor growth and angiogenesis, and 3) To determine the effect of depleting TAM on the outcome of chemotherapy.

BODY

Two tasks are defined in the study: Task 1, Establish optimum conditions for depleting TAM using liposome encapsulated clodronate; and Task 2, Determine effects of macrophage depletion on breast cancer growth and angiogenesis, and the outcome of chemotherapy. Shortly after the funding of this project in September 2003, there was an unexpected turnover of the laboratory personnel, as a result the research in this project did not get started until after April 2004. A request for a no cost, one-year extension of the project was granted in September 2004. Thus, this report represents the first annual report, rather than the final report.

There are 4 stages proposed to accomplish Task 1: a, Establish the culture of 4T1 breast cancer cells and breast cancer model in BALBc mice; b, Prepare liposome-encapsulated clodronate; c, Treat (intra-tumoral injection) tumor-bearing mice with liposome-encasulated clodronate; and d, Process tissue for immunohistochemical staining of macrophage-specific marker and quantify macrophages using Chalkley point counting method.

Two experiments have been carried out to accomplish Task 1. In the first experiment, 45 female mice at 12 weeks of age were obtained and quarantined for 2 week before sc injection of 10^5 cells in 100 μl. The body weight of the animals was recorded every 2-3 days. Tumors became visible around 8 days after 4T1 cell inoculation. The tumor size was measured with a caliper every 2-3 days, and the tumor volume was estimated using the formula $(L \times W^2)/2$ (10-12). Two weeks after the inoculation of 4T1 cells, tumors were well established in 40 mice. The animals were divided into 3 groups: a group with no injection as control, a group with injection of 10 μl liposome-encapsulated clodronate, and a group with injection of 10 μl liposome-encapsulated PBS (empty liposome) into the tumors. At intervals of 1, 3, 5, and 8 days after liposome injection, tumors were removed, fixed in buffered formalin for immuno-staining with macrophage-specific marker to quantify the number of macrophages. Results of tumor volume measurements revealed no significant difference between control and clodronate-injected group that was treated with 10 μl liposome-encapsulated clodronate (Figure 1).

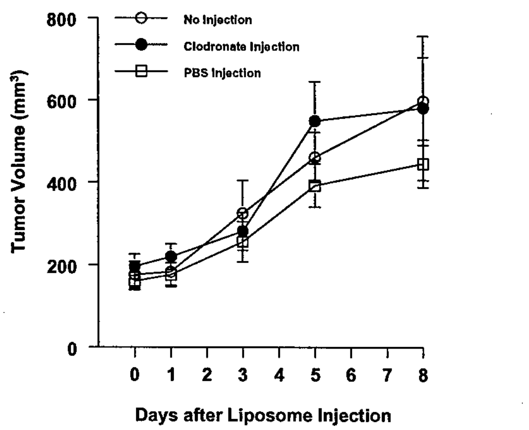

Figure 1. Tumor growth after injection of Clodronate-encapsaled liposome. 40 tumor-baring mice were divided into 3 groups and treated with no injection, clodronate injection, PBS injection, respectively. Day 0 marks the day of injection. Tumor volume was determined as described in the text. Numbers of animals in each group at each data collection day are shown in **Table 1**. Results are presented with mean ± S.D.

Table 1. Numbers of animals in each group

	n				
	Day 0	Day 1	Day 3	Day 5	Day 8
No Injection	14	14	10	7	4
Clodronate Injection	13	13	10	7	4
PBS Injection	13	13	8	5	2

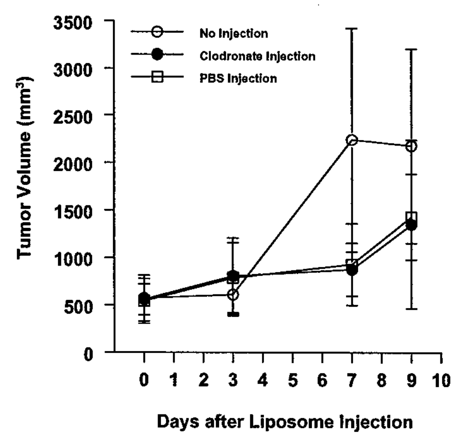

Figure 2. Tumor growth after injection of Clodronate-encapsaled liposome. 28 tumor-baring mice were divided into 3 groups and treated with no injection, clodronate injection, PBS injection, respectively. Day 0 marks the day of injection. Tumor volume was determined as described in the text. Numbers of animals in each group at each data collection day are shown in **Table 2**. Results are presented with mean ± S.D.

Table 2. Numbers of animals in each group

	n			
	Day 0	Day 3	Day 7	Day 9
No Injection	9	9	6	3
Clodronate Injection	9	9	6	3
PBS Injection	10	10	7	4

In the second experiment, a total of 28 mice were obtained and processed as in the first experiment except that 30 μl (15 μl at 2 sites) of liposome-encapsulated clodranate or empty liposome was injected intra-tumorally. Similar to the first experiment, tumors became visible around a week and well established 2 weeks after inoculation. At intervals of 3, 7, and 9 days after liposome injection, tumors were removed, fixed in buffered formalin for immuno-staining with macrophage-specific marker to quantify the number of macrophages. Results of tumor volume measurement showed no significant difference between clodronate-injected group and PBS-injected group (Figure 2). The difference between injected and non-injected group had no statistical significance.

The tumor tissues collected from these two experiments were processed for immunochemical staining. Paraffin-embedded tumor tissues were sectioned into 4-6μm slices. The slides were deparafinated, trypsinized or heated to activate the antigen, and processed in a DAKO Autostainer with an antibody to mouse macrophage-surface marker F4/80, HRP-conjugated secondary antibody, and DAB substrate. The slides were then counter stained with hematoxylin. So far, we have not been able to identify macrophages in these tumor sections. Currently, we are using a different antibody to macrophage markers (anti-Mac-1) attempting to improve macrophage detection.

KEY RESEARCH ACCOMPLISHMENTS

- Established breast cancer model in BALBc mice.
- Treated tumor-bearing mice with liposome-encasulated clodronate.
- Process tissue for immunohistochemical staining with macrophage-specific marker.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

The purpose of this project is to determine whether in vivo depletion of tumor-associate macrophages (TAM) is feasible and if it is feasible, what is the effect of TAM-depletion on the growth of breast cancer and its response to cancer chemotherapy. Using a murine subcutaneous breast cancer model, we have demonstrated that intra-tumoral injection of up to 30 μl of liposome-encapsulated clodronate had no effect on the growth of breast cancer. Whether it has any effect on the depletion of TAM is currently under investigation.

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APPENDICES

None