Advances in Brief

Activation of Omental Milky Spots and Milky Spot Macrophages by Intraperitoneal Administration of a Streptococcal Preparation, OK-432

Masataka Shimotsuma,1 Max W. Simpson-Morgan, Toshio Takahashi, and Akeo Hagiwara

Developmental Physiology Group, Division of Clinical Sciences, John Curtin School of Medical Research, Australian National University, G. P. O. Box 334, Canberra, ACT 2601, Australia [M. S., M. W.-S.-M.], and First Department of Surgery, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602, Japan [M. S., T. T., A. H.]

Abstract

Omental milky spots are omentum-associated lymphoid tissues that cede peritoneal macrophages and participate in the immunity of the peritoneal cavity. We studied the changing surface features of milky spots and milky spot macrophages of Wistar rats, following the i.p. administration of OK-432, a killed streptococcal preparation (1 Klinische Einheit [unit] in 5 ml of phosphate-buffered saline) by the use of scanning electron microscopy. OK-432-activated macrophages demonstrated marked surface membrane activity and migrated through the stomata of the milky spot into the peritoneal cavity. The characteristic features of activated milky spots and milky spot macrophages were noted as early as 3 h following the administration of OK-432, and continued to be observed until 7 days after the injection. By 14 days after the injection, the structural integrity of the milky spot was partially lost. The activation of milky spots and milky spot macrophages by OK-432 provides a convenient in vivo system for the monitoring and study of i.p. cellular events.

Introduction

In recent years, i.p. immunotherapy has become a widely used adjuvant to the postsurgical or palliative treatment of i.p. malignant diseases (1–4) because i.p. administration permits the direct introduction of BRMs2 into the involved areas of the peritoneal cavity. This therapy is based on the theory that optimal BRMs will augment host resistance to malignant tumors by stimulating specific and/or nonspecific immunity (5). While the mechanism of immunotherapeutic activity in the peritoneal cavity has not yet been elucidated in detail, the peritoneal macrophages are recognized as important effector cells in the host defense system against tumors and metastases.

Milky spots in the greater omentum are composed of numerous macrophage and lymphocyte aggregations, and are generally recognized as the site of origin of free peritoneal macrophages, which act as the first line of defense in the peritoneal cavity (6–9). Such in vivo observation of the function and the transformation of milky spots and milky spot macrophages in the greater omentum, following activation by BRMs, is clinically relevant. In this study i.p. administered OK-432, a killed streptococcal preparation, was used as the BRM, and the changes in the surface features of milky spots and milky spot macrophages were studied with the use of scanning electron microscopy.

Materials and Methods

Animals. Inbred Wistar male rats, 4–8 weeks old, were used. All rats were maintained in an air-conditioned room, with water and laboratory chow available ad libitum.

Agents. OK-432 was produced by incubating low virulence, Su type III, group A Streptococcus pyogenes of human origin with penicillin G, followed by lyophilization of the incubation mixture (Chugai Pharmaceutical, Tokyo, Japan). One Klinische Einheit [unit] of OK-432 corresponds to 0.1 mg of dried streptococci. The lyophilized OK-432 was resuspended in PBS for experimental use.

Macrophage Population in Peritoneal Exudate Cells after Injection of OK-432. Before sampling the omentum, we harvested the exudate cells by peritoneal lavage at each time point after the injection of OK-432, and counted the macrophage population in 300 peritoneal exudate cells, using Giemsa stained smear samples.

Identification of Macrophages in Omental Milky Spots. To confirm the macrophages located in the surface layer of the omental milky spots after the injection of OK-432, several methods were used as follows: morphohistochemical characteristics of macrophages with the use of hematoxylin-eosin staining and nonspecific esterase staining of omental milky spot sections, and phagocytic activity of milky spot macrophages by i.p. injection of carbon particles.

Scanning Electron Microscopical Examination. For surface morphological study by scanning electron microscopy, OK-432 1 KE in 5 ml of PBS were injected into the rat peritoneal cavity. As the control group, 5 ml of PBS were injected into the rat peritoneal cavity, in addition to the nontreated control group. Five rats were used in the experimental groups and three rats were used in the control groups at each time point. The greater omentum was harvested after the injection for 3 h, 12 h, 1 day, 3 days, 7 days, 10 days, and 14 days after the injection according to the methods of Dux et al. (10). Six or seven trimmed specimens (about 10 x 10 mm in each specimen) that included milky spots were taken from each rat omentum. Therefore, we sampled at least 30 sections from each experimental group and 18 sections from each control group at each time point. The specimens were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, then postfixed in 1% OsO4 for 1.5 h. After fixation, the specimens were dehydrated in a series of graded acetone solutions for critical-point drying, and then gold coated for scanning electron microscopy (Hitachi H-7000) observation.

Results

In the nontreated rats, the number of milky spots was between 1 and 4 in each trimmed specimen of omentum. The surface observation of the milky spots revealed the aggregation of many macrophages which shared the milky spot surface with mesothelial cells, as shown in Fig. 1a. The milky spot macrophages were round and almost uniform in size (approximately 5 μm in diameter). These macrophages had multiple microvilli but did not show evidence of membrane activity, such as ruffles on their surface. This observation was not basically changed through the control groups with the i.p. injection of PBS at each time point.

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2 The abbreviations used are: BRM, biological response modifier; PBS, phosphate-buffered saline.
In contrast, in the experimental group, the number of milky spots increased between 5 and 12 in each trimmed specimen following the injection of OK-432. Scanning electron microscopy showed that as early as 3 h following the injection of OK-432, the number of macrophages increased on the surface of the milky spots, and by day 1 after the injection of OK-432, the milky spot was almost completely covered by macrophages. The size and shape of the milky spot macrophages were conspicuously variable. Macrophages appeared larger (up to 12 μm in diameter) and exhibited exaggerated cell projections and ruffles (Fig. 1b). Furthermore, these macrophages were noted to appear through the intercellular gap (stomata) of the mesothelial cells of the milky spot (Fig. 1c). Occasionally, prominent membrane activity was observed on the surface of the macrophage in the process of migrating through the stomata of the milky spot (Fig. 1d). Although in the control group it was very rare to find a macrophage appearing through the stomata of a milky spot, in the experimental groups, especially at 12 h, 1 day, and 3 days after the injection of OK-432, at least two or three macrophages were found to be appearing through the stomata of a milky spot. In addition, the mesothelial cells which covered the milky spots were similarly influenced by OK-432, and their microvilli increased in both prominence and number. These changes were apparent up to 7 days after the injection of OK-432. However, by 14 days after the injection the proportion of macrophages began to decrease, the continuity of the mesothelial cells of the milky spots was interrupted, and the structural integrity of the milky spot was partially lost.

The macrophage population in 300 peritoneal exudate cells increased from 3 h (8.4%) after the injection of OK-432, reached its peak on 3 days (42.7%), and also continued to 7 days following the injection (34.2%).

Discussion

The i.p. administration of OK-432 is known to activate peritoneal macrophages and to stimulate antitumor activity in the peritoneal cavity (11). While the activation of macrophages by OK-432 has been studied by many researchers (11–15), observations have typically been performed in vitro using peritoneal exudate macrophages. Omental milky spots, the most abundant elements of which are macrophages, are omentum-associated lymphoid tissues that participate in the immunity of the peritoneal cavity (6–9). In a normal steady state, the majority of the milky spot macrophages are resident cells. However, under inflammatory conditions, many exudate macrophages occupy the milky spots and migrate into the peritoneal cavity following increased microvascular permeability in the milky spots (16, 17). Through observation of the milky spots, the present study has shown that the i.p. administration of OK-432 activates the peritoneal macrophages and influences their surface morphology in vivo.
In the mouse, the i.p. administration of OK-432 was noted to cause the induction of macrophages in the peritoneal cavity 4 h later, with maximal induction observable at 16 h. The peritoneal exudate macrophage of mice 3 days after i.p. injection of OK-432 showed both maximum cytotoxic factor production on restimulation with OK-432 in vitro and maximum cytotoxic activity against the tumor in vitro (18). In the present study, OK-432 stimulated the activation of milky spot macrophages as early as 3 h following administration, and the OK-432-activated macrophages demonstrated marked membrane activity on their surface, recognizable in the milky spots up to 7 days after the OK-432 injection. In the mouse, Daems and Koerten (17) demonstrated that the number of macrophages had increased significantly 16 h after the i.p. injection of various stimulants, such as proteose peptone, and invariably remained elevated until 8 days after the injection. Dux (19) noted that the surface of mouse milky spots became bumpy and rough 4 h after the i.p. injection of sheep RBC, and collapsed at 8 days after the injection. Our observations correspond well with these earlier observations, and confirm the significant value of milky spots as a source of macrophages in the peritoneal cavity.

Macrophages apparently form clusters near the peritoneal surface of the milky spots and are oriented toward the peritoneal cavity for migration, whereas clusters of B-cells and T-cells are typically found in periarteriolar locations within the milky spots (6, 7). Such cell zonation would facilitate the phagocytosis and processing of antigens which have found access to the peritoneal cavity. Although earlier ultrastructural examinations have suggested that the macrophages may migrate through the stoma of the milky spot into the peritoneal cavity as free peritoneal macrophages (16, 17, 20, 21), this is the first report of macrophages with prominent ruffled membranes in the process of migrating through the stoma of the milky spot into the peritoneal cavity as shown in Fig. 1d.

Several recent in vitro experimental studies have suggested that the cytotoxic activity of macrophages, following activation by BRMs, correlates with their surface morphology, ruffle formation, and polar spreading (12, 13, 15). Fujita (13) reported that the majority of activated macrophages show marked ruffle formation and polar spreading, demonstrating notable cytotoxic activity against the tumor cells, with peak activity evidenced at 4 days after the i.p. administration of OK-432. In the present in vivo study, spreading macrophages with polarity were not observed in the milky spots even after OK-432 activation. This discrepancy may be due to differences in the in vivo versus in vitro appearance of macrophages, as mentioned earlier by Orenstein and Shelton (22).

Milky spots have been described not only as regions of resident macrophage development and release, but also as exit pathways to the regional lymph nodes (23). Therefore, i.p. immunotherapy might constitute a useful method of augmentation of regional lymph node immunity directed against tumor cells via the activation of milky spot macrophages.

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