Limited Capability of Regional Lymph Nodes to Eradicate Metastatic Cancer Cells

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ABSTRACT

The capacity of lymph nodes to eradicate cancer is a controversial issue. The purpose of this study was to determine the interplay between tumor growth and host resistance at early stages of lymph node metastasis. A metastasis model was made in the rat mesenteric lymph node, and migration of cancer cells was visualized in vivo. The lymph node was removed for histologic analysis and cytokine measurement. Migrant cancer cells were initially arrested in the marginal sinus. After an initial increase, the number of cancer cells in the marginal sinus declined until 48 hours after inoculation. Germinal centers and lymphoid cells in the medulla proliferated before 48 hours. ED3+ macrophages incorporated apoptotic cancer cells, but significant cancer proliferation occurred after 4 days. lymph nodes depleted of macrophages were massively invaded by cancer cells. Tumor necrosis factor α and interleukin (IL)-1β in the nodes transiently increased after 1 hour and 3 hours, respectively, and were expressed in ED3+ and ED2+ macrophages, respectively. These changes were followed by a transient increase in IL-2. Interferon-γ and IL-12 did not increase during the early stages of metastasis, but they decreased after 48 hours. In conclusion, the marginal sinus constitutes a mechanical barrier against cancer cell passage. Early pathological manifestations in the regional lymph node are consistent with those in cancer patients with improved survival. Parasinus macrophages play a role in the transient antimetastatic capability of the node, and cytokines secreted by these cells increased at the early stages of metastasis. Deterioration of cytokine induction may be responsible for subsequent cancer proliferation.

INTRODUCTION

Regional lymph nodes are the most common and earliest site of metastasis of epithelial tumors, and the possibility that the lymph node is an initial filter for cells from the primary tumor has long been debated (1–4). The barrier function of the lymph node can be divided into two categories (4, 5). Hence, the lymph node acts as a mechanical barrier to prevent passage of tumor cells though the node and also acts as a biological barrier to inhibit tumor growth in the node. The dramatic clinical success of sentinel node navigation surgery (6, 7) suggests that the regional lymph node has an effective filtering function as a mechanical barrier against migrating cancer cells. On the other hand, the presence of even micrometastases in regional lymph nodes correlates with a poorer prognostic outcome (8). Whether all of the regional lymph nodes should be removed, irrespective of their status in terms of metastatic involvement, remains a controversial issue. This is because the biological capacity of lymph nodes to eradicate migrate cancer cells is understood only in a fragmentary fashion. For example, it is not known which lymph node cells play a key role in the mechanical or biological barrier against lymphogenous metastasis.

A small number of tumor cells seem to be rejected in the regional lymph node in situ (9, 10). Clearly, the biological responses of lymph nodes during the process of metastasis are based on their immune responses (9–11). On the other hand, these immune responses seem to be inadequate to prevent lymph node metastasis. A recent clinical study indicated that antitumor immune functions of sentinel lymph nodes are down-regulated by tumor-derived products (12). The reaction pattern in the regional lymph nodes provides information on the type of immunologic response and on patient prognosis. The pathology of regional lymph nodes indicates that sinus histiocytosis or hyperplasia of follicles with prominent germinal centers reflects favorable stigmata for improved prognosis (13). However, it is very difficult to obtain information on the functional interactions of regional lymph node cells with metastatic cancer cells, especially at the initial stage of human tumor metastasis. To analyze the interplay between the tumor and host immune surveillance at early stages of metastasis, establishment of an experimental lymph node model in an animal with immunocompetent cells is required.

The differential immune response is largely dictated by the expression and secretion of cytokines. Lymph node are cytokine-rich environment. In the lymph node, T cells, B cells, macrophages, dendritic cells, and natural killer cells secrete a variety of cytokines, and lymphocytes in lymph nodes of tumor-bearing animals are capable of mounting an immune reaction against tumor cells when the lymphocytes are stimulated by cytokines in vitro (14). Administration of tumor-infiltrating lymphocytes and interleukin (IL)-2 has been shown to have negative effects on tumor growth in human studies (15, 16). Hence, it is possible to modify the metastatic process by artificial manipulation of the cytokine environment, but the cytokine profile in the regional lymph node microenvironment at early stages of metastasis has yet to be documented.

The first aim of this study was to establish an experimental metastasis model in the central lymph node to study interplay between host cell immunity and cancer growth at early stages of metastasis. Because we aimed to study host immunity, we did not use immunodeficient animals but used rats with normal immunity. Cancer cells were injected into the rat cecum submucosa to create metastasis in the meso-cecum lymph node, which corresponds to the sentinel lymph node of the cecum. We then followed a time course of growth and decay of the tumor and the host defense. The second aim was to study in vivo whether the regional lymph node truly has a mechanical filtering capability, through direct visualization of the behavior of migrating cancer cells in the meso-cecum lymph node. The third aim was to investigate changes in the cytokine profile of the lymph node at early stages of metastasis. Fourthly, we focused on the role of macrophages in cancer cell elimination because they seem to be the major source of cytokines and they reside around the lymph node sinus at the place of entry of cancer cells.

MATERIALS AND METHODS

Cancer Cell Preparation. AH130 rat ascites hepatoma cells were kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). These cells are highly metastatic to the lymph node (5) and liver (17) of rats. AH130 cells were maintained by serial intraperitoneal implantation in male Donryu rats every 7 days, and the ascites was used in the experiments on the 7th day of passage. The cells in the ascites were washed three times with RPMI 1640 supplemented with 10% fetal calf serum. The cancer cell suspension was then placed in a culture dish for 15 minutes at 37°C.
under 5% CO₂/95% O₂ to eliminate contaminated peritoneal macrophages, which adhered to the dish during incubation. The cancer cells were collected by gently aspirating the suspension. The final cell concentration was 10⁷ cells per mL of RPMI 1640, and cell viability was >98%, as assessed by trypan blue exclusion. AH130 cells in the suspension were in a single cell state, and this greatly facilitated injection of the cell suspension.

Animal Preparation. Male Donryu rats weighing 250 to 300 g were used in the study. All animal protocols were approved by the Keio University Animal Research Committee. The animals were maintained on a standard laboratory chow diet and had access to tap water ad libitum. The rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal), and the abdomen was opened via a midline incision. The cecum was exteriorized for cancer cell inoculation, allowing easy identification of a meso-cecum lymph node, the regional lymph node of the cecum. Approximately 0.05 mL of the cancer cell suspension was injected into the cecum submucosa using a micropipette with a 30-gauge needle. Because a meso-cecum lymph node drains two to three affluent lymphatic vessels from the cecum, the injection was performed at four different sites along the mesocolon of the cecum. At various time points after injection, the rats were sacrificed using an ether overdose, and the meso-cecum lymph nodes were removed and weighed.

Preparation and Injection of Liposomes for Macrophage Depletion and Activation. Multilamellar liposomes were prepared according to the method of Van Rooijen and Sanders (18). Briefly, 8 mg of cholesterol (Sigma, St. Louis, MO) were dissolved in 10 mL of chloroform in a 500-mL round-bottomed flask, and then 0.86 mL of a 100 mg/mL solution of egg yolk lecithin was added. The chloroform phase was removed by low vacuum rotary evaporation, and a thin phospholipid film formed against the inner wall of the flask. The phospholipid film was dispersed in 10 mL of PBS containing 0.6 mol/L dichloromethyleneephosphonic acid (DMDP; Sigma) or 4 mmol/L N-acetyl muramyl-l-alanyl-o-isoglutaminine [NAAI (also called muramyl dipeptide); Sigma], and the suspension was sonicated in a water bath sonicator for 3 minutes. The suspension was kept at room temperature for 2 hours under nitrogen gas. After gentle shaking, the suspension was sonicated in a water bath sonicator for 3 minutes. The suspension was then kept under nitrogen gas for an additional 2 hours at room temperature to allow swelling of the liposomes. Nonencapsulated agent was removed by centrifugation (10,000 × g), and the multilamellar liposomes containing the agent were washed three times using sterilized PBS with centrifugation at 25,000 × g. Finally, the pellet was resuspended in 4 mL of sterilized PBS.

For depletion or activation of macrophages in the meso-cecum lymph node, rats were pretreated with liposomes containing DMDP (18) or liposomes containing NAAI (19, 20). About 0.05 mL of the liposome suspension in PBS was injected with a 30-gauge needle into four different regions of the submucosa of the posterior wall in the rat cecum. The cancer cells were inoculated into the submucosa of the anterior wall 48 hours after treatment with liposome-DMDP or liposome-NAAI.

In vivo Fluorescence Microscopy. For fluorescent labeling of cancer cells, carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) was dissolved in dimethyl sulfoxide to give a 1.56 mmol/L solution, and a small aliquot (300 µL) was stored in a sealed cuvette under argon gas at −80°C until the experiments were performed. Cancer cells were incubated in a carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) was dissolved in dimethyl sulfoxide to give a 1.56 mmol/L solution, and a small aliquot (300 µL) was stored in a sealed cuvette under argon gas at −80°C until the experiments were performed. Cancer cells were incubated in a carboxyfluorescein diacetate succinimidyl ester solution (20 µL of stock solution was diluted with 20 mL RPMI 1640) for 30 minutes at 37°C. The labeled cancer cells were immediately centrifuged through a cushion of heat-inactivated fetal bovine serum and washed twice in cold suspension medium. The cells were resuspended in the medium at a concentration of 10⁷ cells per mL and injected into the cecum submucosa with a 30-gauge needle. Fluorescence intensity of the labeled cells decayed gradually in the culture dish, but >95% of the cells could be visualized by fluorescence microscopy after 48 hours in culture.

The meso-cecum lymph node was dissected carefully to visualize the surface of the node and affluent lymphatic vessels. For visualization of the effluent lymphatic vessels, the lymph node was inverted carefully to expose the hilus of the node. At various time points after injection of fluorescent cancer cells, the lymph node was epi-illuminated and observed with a fluorescence microscope. Epi-illumination was provided by a 100-W mercury DC lamp source with a Nikon exciter filter (400–440 nm), a Nikon dichroic mirror (455 nm), and a Nikon barrier filter (470 nm). The area was visualized and digitized using a color chilled charge-coupled device camera (Hamamatsu Photonics, Shizuoka, Japan), and the image was recorded on a SVHS videotape and MO disk. For fluorescence staining of the wall of affluent and efferent lymphatic vessels, 0.1% rhodamine 6G (Sigma) in physiologic saline was applied topically to the mesentery and lymph nodes.

Antibodies Used for Immunohistochemistry. The primary antibodies used for determining macrophage and dendritic cell distribution in the regional lymph node were mouse antirat macrophage ED2, mouse antirat macrophage ED3, and mouse antirat OX-62 (Srotec Ltd., Oxford, United Kingdom). The antibodies used for analyzing cytokine localization in the lymph node were rabbit antirat tumor necrosis factor (TNF)-α and rabbit antirat IL-1β (Yanaihara Institute Inc., Fujinomiya, Japan). Rabbit antimacrophage migration-inhibitory factor (MIF) was purchased from Abcam Limited (Cambridge, United Kingdom). Rabbit antikeratin for wide spectrum screening (DAKO, Carpinteria, CA) was used to detect cancer cells in the lymph node. Rabbit anti–single-stranded DNA and mouse antirat Ki67 (DAKO) were used to determine apoptotic and proliferating cells, respectively. The secondary antibodies used were alkaline phosphatase (ALP)-conjugated sheep anti-mouse IgG, ALP-conjugated goat antirabbit IgG (Sigma), and horseradish peroxidase-conjugated goat antimouse or antirabbit IgG (EnVision +; DAKO).

Histologic and Immunohistochemical Analysis. For histologic analysis, meso-cecum lymph nodes were fixed in 10% neutral buffered formalin, and paraaffin-embedded tissue sections (4 µm) were stained with hematoxylin and eosin (H&E) using standard techniques. For immunohistochemistry, paraaffin sections or cryostat sections were used. Staining for OX-62 was performed in cryostat tissue sections, whereas staining for ED3 was performed in both cryostat and paraaffin-embedded sections. Other antibodies were stained in paraaffin-embedded sections. To prepare cryostat sections, lymph nodes were freshly frozen in Tissue-Tek OCT compound (Sakura Finetechanical, Tokyo, Japan). Cryostat sections (4–8 µm) were fixed in pure acetone at room temperature for 10 minutes and dried. Paraaffin-embedded sections were deparaffinized, and target retrieval was performed. For retrieval of ED2, ED3, TNF-α, IL-1β, and keratin antigens, proteinase K (DAKO) was topically applied to the deparaffinized sections for 6 minutes. For antigen retrieval of Ki67 and MIF, the deparaffinized sections were heated in citrate buffer in an autoclave for 10 minutes. No antigen retrieval procedure was performed for single-stranded DNA staining.

For double immunostaining, the sections were incubated in 0.3% H₂O₂ in methanol for 10 minutes to inactivate endogenous peroxidase and washed with PBS containing 0.1% Tween 20 (PBST). After incubation with blocking solution (Block Ace; Dainippon Seiyaku, Tokyo, Japan) for 10 minutes, sections were incubated with a primary antibody for 1 hour at room temperature. Thereafter, each step was followed by washing three times with PBST for 3 minutes. The bound antibody was detected with DAB-conjugated IgG for 40 minutes. The labeled cells were colored red or blue with ALP substrate kit I or III (Vector red or blue; Vector Laboratories Inc., Burlingame, CA). Sections were then incubated with the secondary antibody for 1 hour, followed by incubation with horseradish peroxidase-conjugated IgG for 40 minutes. The labeled cells were colored brown with 3,3′-diaminobenzidine hydrochloride (DAB), using a DAB reagent set (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD).

Single immunostaining was performed using the indirect immunoperoxidase phospahate or immunoperoxidase method, as described above. In the tissues colored with DAB, the slides were counterstained in Mayer’s hematoxylin.

The number of Ki67-positive proliferating cells was determined using a grid over an eyepiece and a ×40 objective and expressed relative to the total number of cells within eight random high-power fields in the medullary cord. More than 80 cells were counted in each field area of 9 mm².

Cytokine Measurement. A whole lymph node was homogenized in ice-cold lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.2), 10 mmol/L EDTA, and 1% Triton X-100 containing protease inhibitor mixture (Boehringer Mannheim GmbH, Mannheim, Germany)]. Debris was removed by centrifugation, and the supernatant was used as the whole lysate. The TNF-α, IL-1β, IL-2, interferon (IFN)-γ, IL-4, and IL-12 levels in the supernatant were measured in duplicate, using commercially available enzyme-linked immunoabsorbent assay kits (BioSource International Inc., Camarillo, CA). The concentrations of each cytokine were expressed as the amount of cytokine per tissue weight.

Statistical Analysis. Results were evaluated by the Mann-Whitney U test (two-tailed). Probability values of <0.05 were considered significant.
RESULTS

Formation of Lymph Node Metastasis. Fig. 1A shows the enlargement of a meso-cecum lymph node on day 7 after inoculation of cancer cells. From 4 days after inoculation, gross enlargement of the lymph node became apparent, and the weight of the lymph node had increased significantly 4 and 7 days after inoculation (Fig. 1B). This enlargement could be due to either hyperplasia of lymph node cells or growth of tumor cells. Thus, we analyzed H&E-stained sections of the node to determine the metastasis index, based on histologic findings of cancer invasion of the lymph node (ref. 5; Fig. 1C). Similar to the time course of the increase in lymph node weight, the metastasis index increased 4 and 7 days after inoculation (Fig. 1D). The rats had cancerous peritonitis after 14 days, and most died before 21 days. On day 7, most of the regional lymph nodes showed grade 2 or 3 changes, with invasion of cancer cells over the inner linings of the marginal sinus to the cortex and paracortex or formation of cancer nests. On day 4, most of the regional lymph nodes revealed grade 1 invasion, showing marginal proliferation of cancer cells surrounding the lymph node parenchyma (Fig. 1E). The cancer cells appeared to have advanced over the marginal sinus, but in fact they were confined within the marginal sinus, as shown by silver impregnation staining, which clearly identified the inner lining of the sinus endothelium (Fig. 1F).

Migration and Arrest of Cancer Cells in Regional Lymph Nodes at Early Stages of Metastasis. The afferent lymphatic vessels in the rat mesentery, which are approximately 100 to 250 \( \mu \text{m} \) in diameter, showed contractile activity at 7 to 20 cycles/min, propelling lymph fluid toward the draining lymph node (Fig. 2B). The meso-cecum lymph node drains two to three afferent lymphatic vessels to its marginal sinus from the cecum. With in vivo microscopy, the terminal of the afferent lymphatic vessel appears like a club end and opens to the surface of interfollicular areas of the lymph node (Fig. 2C). Within 5 minutes after injection of fluorescent cancer cells into the cecum submucosa, the cells reached the meso-cecum lymph node via afferent lymphatic vessels (Fig. 2D). Cancer cells intermittently flowed out in a radial pattern from the termini of the afferent lymphatic vessels, and the rhythmic flow of cancer cells seen in the marginal sinus was synchronized with the contraction of the afferent lymphatic vessels. The cancer cells were then arrested somewhere in the marginal sinus.
Fig. 2. Arrival of cancer cells at the meso-cecum lymph node, observed by in vivo fluorescence microscopy (B–F and J) and immunohistochemistry (G–I and K–N). The scale bars indicate 100 μm. A, schematic representation of the lymph node, indicating the location of the images in B–F. B, afferent lymphatic vessels in the mesentery, showing contraction movements (arrows). The walls of the lymphatic vessels appear white, whereas the blood vessel appears black. Lymph fluid flowed toward the lymph node. C, the surface of the lymph node, consisting of follicles, and the terminal of an afferent lymphatic vessel (arrow). D, a meso-cecum lymph node 5 minutes after injection of fluorescence-labeled cancer cells into the cecum submucosa. The cancer cells flowed through the afferent lymphatic vessel (arrowheads). Cancer cells intermittently flowed out in a radial pattern from the terminal (t) of the afferent lymphatic vessel. At the top right, fluorescent cancer cells can be seen emerging from a different afferent lymphatic vessel. E, a meso-cecum lymph node draining cancer cells 30 minutes after injection of fluorescence-labeled cancer cells into the cecum submucosa, seen at higher magnification. The rhythmic flow of cancer cells was synchronized with contraction of the afferent lymphatic vessels. The cancer cells were then arrested in the marginal sinus. F, efferent lymphatic vessels (e) in the hilus of the lymph node 30 minutes after injection of fluorescence-labeled cancer cells. The efferent lymphatic vessels showed similar contraction movements to the afferent lymphatic vessels and drained lymph fluid from the medullary sinus (me), but injected cancer cells were not seen in the medullary sinus or in the efferent lymphatic vessels. G, cryostat section showing immunostaining of ED3+ macrophages, which were distributed beneath the marginal sinus (ma) and medullary sinus (me). H, 1 hour after injection, fluorescent cancer cells (white) were arrested in the marginal sinus, where they made contact with ED3+ macrophages, but they did not reach the medullary sinus (me). I, immunostaining of AH130 cancer cells in the marginal sinus, showing expression of macrophage MIF. J, time course of the change in the number of fluorescent cancer cells seen in the marginal sinus. Each data point represents the mean ± SE of results in eight lymph nodes. *, significantly different at P < 0.05. K–M, cancer cells in the marginal sinus 1 hour, 6 hours, and 48 hours after injection. The time course of the change in the number of cancer cells in the marginal sinus demonstrated by immunohistochemistry was consistent with those demonstrated using in vivo fluorescent microscopy. The cancer cells were stained with an antikeratin antibody. N, double-color immunostaining showing ED3+ macrophages (blue) and keratin-positive cancer cells (brown) 12 hours after inoculation of cancer cells in the medulla. Some cancer cells were incorporated by macrophages.
B cells in the germinal center. *, significantly different at *P < 0.05.

Fig. 3. Pathological findings in regional lymph nodes, showing germinal center proliferation at early stages of metastasis. A, cancer cells (arrows) in the marginal sinus 1 hour after inoculation. B, a serial section of the lymph node stained with H&E, showing an increase in follicles with germinal centers. C, a serial section stained with Ki67, showing proliferation of B cells in the germinal center. D, time course of the number of follicles with germinal centers. Each data point represents the mean ± SE of results in six lymph nodes. The follicles with germinal centers significantly increased 3 and 12 hours after inoculation, compared with the control (0 hours). *, significantly different at *P < 0.05.

Pathological Changes in Regional Lymph Nodes. Hyperplasia of follicles was observed as an initial pathological change in the regional lymph nodes before cancer cell migration. There were few germinal centers in the meso-cecum lymph nodes after the arrival of cancer cells. These macrophages were intermingled with cancer cells in the marginal sinus. Many apoptotic bodies were found at the boundary between clusters of cancer cells and the ED3+ macrophages (Fig. 5D). In lymph nodes that were almost replaced by tumor cells after 7 days, few signs of sinus histiocytosis were found. Most of these histiocytes consisted of ED3+ macrophages (Fig. 5B), and some ED3+ macrophages were intermingled with cancer cells in the marginal sinus. Many apoptotic bodies were found at the boundary between clusters of cancer cells and the ED3+ macrophage population (Fig. 5C). These apoptotic bodies, which were probably apoptotic cancer cells, were phagocytosed and engulfed by the macrophages (Fig. 5D and E). In contrast, no accumulation of dendritic cells occurred. These cells were found only in the vicinity of the cancer nest and in smaller numbers than the macrophages (Fig. 5F). Macrophages accumulated at the primary injection site in the cecum submucosa and had surrounded the cluster of cancer cells after 6 hours, with no cancer cell invasion of the lymphatic vessels (data not shown).

Effect of Depletion or Activation of Macrophages on Lymph Node Metastasis. We depleted macrophages to determine whether they counteract or aggravate metastasis. Pretreatment with liposomes containing DMDP resulted in depletion of both ED2+ and ED3+ macrophages in the lymph nodes (Fig. 6A and B), which were massively invaded by cancer cells in the cortex and paracortex (Fig. 6C).
These lymph nodes also significantly increased in weight after inoculation, compared with lymph nodes with a normal macrophage population (Fig. 6D), and showed significant elevation of the metastasis index (Fig. 6E). On the other hand, the weight and metastasis index of lymph nodes with macrophages activated by pretreatment with liposomes containing NAAI were significantly lower 7 days after inoculation, compared with those for lymph nodes that did not undergo pretreatment (Fig. 6F and G).

**Cytokine Profile in Regional Lymph Nodes.** Fig. 7A shows the time course for changes in cytokine concentrations in the meso-cecum lymph node after inoculation. TNF-α and IL-1β in the lymph node significantly increased after 1 hour and 3 hours, respectively, and IL-2 increased significantly during the period from 6 to 24 hours after inoculation. These cytokines returned to basal levels thereafter. IFN-γ, IL-4, or IL-12 did not increase in the earlier periods but significantly decreased during later periods of metastasis. Depletion of lymph node macrophages by pretreatment with liposomes containing DMDP suppressed transient increases in TNF-α, IL-1β, and IL-2 in the lymph nodes after cancer cell inoculation (Fig. 6F and G). The profiles of IFN-γ, IL-4, and IL-12 were unchanged by pretreatment with DMDP. Activation of lymph node macrophages by pretreatment with liposomes containing NAAI did not alter the cytokine profiles after cancer cell inoculation (Fig. 7C), in comparison with no pretreatment of the lymph nodes (Fig. 7A).

Fig. 8 shows the localization of TNF-α and IL-1β expression 3 hours after inoculation in the specific macrophage populations of the regional lymph nodes. A small number of TNF-α-positive cells were seen in the cortex before cancer inoculation (data not shown). After influx of cancer cells into the marginal sinus, ED3+ macrophages expressed TNF-α (Fig. 8A). On the other hand, IL-1β was colocalized with ED2+ macrophages around the medullary sinus (Fig. 8B).

**DISCUSSION**

Despite the fact that the fate of proliferating cancer cells in regional lymph nodes is of decisive importance to disease outcome, the underlying mechanisms that regulate this process remain virtually unknown. In this study, an experimental model of lymph node metastasis was established to investigate the time course of changes in cancer cell growth and immune reactions in the regional lymph nodes at early stages of metastasis. In drawing our conclusions, we note that this lymph node metastasis model has several characteristics that are not
consistent with spontaneous lymph node metastasis. Hence, in this model, the cancer cells reach the regional lymph node within 5 minutes after injection into the cecum submucosa and initially arrive at the lymph node as emboli, due to an increase in local fluid and pressure caused by injection into the cecum submucosa, although thereafter they reach the node through lymph flow inafferent lymphatic vessels. Thus, injected cells have not been selected for immune tolerance at the primary site.

The present study permitted direct visualization of cancer cells migrating from the cecum through afferent lymphatic vessels in the mesentery and entering the marginal sinus of the regional lymph node. The cancer cells were arrested in the marginal sinus after flowing for a certain distance in the sinus, and despite the continuous flow of lymph fluid to efferent lymphatic vessels through the medullary sinus, the cancer cells did not initially reach the medullary sinus or efferent lymphatic vessels in the hilus of the regional lymph node. This indicates that the marginal sinus plays a central role in the mechanical filtration capability of regional lymph nodes that acts against cancer cell passage. The mechanism of cancer cell arrest still cannot be fully explained, but it is likely that adhesion molecules on the surface of cancer cells and the endothelium of the marginal sinus play a role in the arrest of the cancer cells (21–23).

Another possible mechanism is entrapment of cancer cells by reticular fibers distributed in the sinus (24) and by macrophages beneath the sinus (25). Among the various cells in the rat lymph node, ED3⁺ macrophages located beneath the marginal sinus are the first to encounter cancer cells. Cancer cells were seen to be in close contact with ED3⁺ macrophages, which protrude as pseudopods into the sinus. It seems likely that contact with each other, certain signals are exchanged between the cancer cells and macrophages. Although a detailed understanding of the process will require additional studies, the cancer cells used in this study expressed MIF, suggesting that the arrival of cancer cells at the sinus could modulate ED3⁺ macrophage migration (26). In human lymph node metastases of prostate cancer, MIF mRNA expression was augmented (27), and recent reports have indicated that MIF promotes tumorigenesis (28, 29). Thus, it is possible that MIF expressed in cancer cells in this study contributes to the progression of the metastatic process.

Cancer cell growth was confined within the marginal sinus until day 4 after inoculation, and only when the cancer cells had completely occupied the marginal sinus did they begin to invade the cortical pulp for the first time. The following phase was characterized by inundation of tumor cells via the marginal sinuses and formation of colonies, with completion of the process of metastasis by 7 days after inoculation. The finding that the marginal sinus is the initial site of cancer cell proliferation in the regional lymph node is consistent with previous reports (4, 5, 9, 10, 25). Proliferation of cancer cells after inoculation is probably due to clonal expansion of cancer cells that are resistant to the host defense. Another possible explanation is that continuous recruitment of cancer cells from the primary tumor to the regional lymph node establishes lymphogenous metastasis (3). In this study, however, few cancer cells flowed from the cecum more than 6 hours after inoculation. Macrophages accumulated at the primary injection site in the cecum submucosa after 6 hours. Invasion of the lymphatic vessels by viable cancer cells was rarely observed, and most of the injected cancer cells became necrotic, resulting in a granuloma after 4 days. Thus, a constant supply of cancer cells to the regional lymph node was not expected. Even bolus intralymphatic injection of cancer cells can complete the metastasis process in the regional lymph node (4).

After an initial increase in number, the cancer cells in the marginal sinus decreased in the period from 12 to 48 hours after inoculation. This transient reduction during the early phases of metastasis has been observed in other animal models (4, 10). In this study, the cancer cells appeared in the medulla from 6 to 48 hours after inoculation, and
some were incorporated by ED3\(^+\) macrophages. Foreign bodies arriving at the lymph node are all incorporated by ED3\(^+\) macrophages beneath the marginal sinus (30), and these foreign body-laden macrophages then migrate to the medullary sinus. Although the detailed mechanism of the transient decrease in cancer cells in the marginal sinus is unknown, possible explanations include migration of cancer cell-incorporating macrophages to the medulla and, after initial arrest in the marginal sinus, migration of cancer cells to the medullary sinus and subsequent phagocytosis by macrophages.

The spontaneous regression of cancer cells in the marginal sinus may depend not only on the immunogenicity of the tumor itself but primarily on the mode and intensity of the initial immune response that is provided by the regional lymph nodes. Reactive histologic changes in regional lymph nodes may be interpreted as morphologic correlates of various

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**Fig. 7. Changes in cytokine profiles of regional lymph nodes after cancer inoculation.** The cytokine concentrations in the lymph node tissue were determined by enzyme-linked immunosorbent assay. Results represent the mean ± SE of cytokine concentrations in six lymph nodes. \(*\) significantly different from control (0 hours) at \(P < 0.05\). A. Proinflammatory cytokines TNF-\(\alpha\), IL-1\(\beta\), and IL-2 increased at early stages of metastasis, whereas IFN-\(\gamma\), IL-4, or IL-12 did not increase in the early period but decreased significantly at the later stage of metastasis. B, cytokine profiles after cancer cell inoculation in lymph nodes pretreated with liposomes containing DMDP. Depletion of lymph node macrophages by DMDP suppressed transient increases in TNF-\(\alpha\), IL-1\(\beta\), and IL-2 at early stages of metastasis. C, cytokine profiles after cancer cell inoculation in lymph nodes pretreated with liposomes containing NAAI for activation of macrophages.
functional immunostages. In this study, follicular hyperplasia and lymphocyte proliferation were induced at early stages of metastasis, and lymphopoiesis in the medullary cord and germinal center has been reported in rats bearing autochthonous breast cancers (11). These responses in experimental models of lymph node metastasis may be related to histopathological findings in regional lymph nodes in breast cancer patients with good prognosis (13). In the present study, these immunoreactions were transiently observed only at early stages of metastasis and diminished thereafter. Sinus histiocytosis, which reflects accumulation of ED3⁺ macrophages and is another clinical manifestation of regional lymph nodes that is associated with improved prognosis, was evident on day 4, at which time the lymph node weight significantly increased with proliferation of cancer cells in the marginal sinus, resulting in expansion of the sinus. At this stage, ED3⁺ macrophages intermingled with cancer cells, and many apoptotic bodies were present at the boundary between the proliferating cancer cells and the accumulating ED3⁺ macrophages. These apoptotic bodies were probably not derived from macrophages but from cancer cells because ED3⁺ macrophages were able to incorporate them. It is evident that the ED3⁺ macrophage lining constitutes a first line of defense against cancer invasion, because (a) cancer nests were formed in the lymph node parenchyma after cancer cells filled the marginal sinus, (b) elimination of macrophages resulted in early and massive invasion of cancer into the lymph node parenchyma, and (c) activation of macrophages partially prevented lymph node metastasis. This initial proliferation of cancer cells in the marginal sinus might be a key for early diagnosis and for targeting metastatic lymph nodes with selective treatment. In contrast, dendritic cells, another antigen-presenting cell population in the lymph node, did not accumulate in the marginal sinus but were distributed only around the cancer nest, and in smaller numbers than macrophages. This result may correlate with the finding of a decrease in dendritic cells in human sentinel lymph nodes of a tumor-bearing host (12).

The capacity of a lymph node to eradicate migrant cancer cells, if any such capacity exists, is limited to the very early stages of metastasis. After this period, the immune reaction directed against the cancer cells in the regional lymph node is beginning to break down. We tried to clarify this deterioration of antimetastatic immune capability from the perspective of the cytokine and macrophage responses. A lymph node consists of numerous lymphocytes, macrophages, and dendritic cells that contain and secrete a variety of cytokines. This cytokine-rich microenvironment seems to be responsible for the antimetastatic capability of regional nodes because many reports indicate that certain cytokines counteract cancer growth (14–16, 31, 32). Barth et al. (33) demonstrated that tumor-infiltrating lymphocyte cultures secrete IFN-γ and TNF-α when stimulated with tumors in vitro and that IFN-γ has direct tumoricidal effects on tumor cells. Although tumor-draining lymph nodes in animals harbor lymphoid cells with antitumor reactivity, they are not capable of mediating regression in adoptive immunotherapy and require further in vitro activation with IL-2 to differentiate into functional effector cells (14). In the interplay between cancer cells and lymph node cells, the capability of the host defense to eliminate cancer growth seemed to deteriorate 48 hours after inoculation in this study. A lymph node microenvironment deficient in cytokines might be incapable of recruiting the appropriate number of cytotoxic lymphocytes, and this may permit cancer cell invasion and growth. Of the cytokines measured, proinflammatory cytokines TNF-α and IL-1β transiently increased after migration of cancer cells to the lymph node, and this was followed by a transient increase in IL-2 from 6 to 24 hours after cancer cell migration. This temporary induction of cytokines may be related to transient histopathological changes, including increases in germinal centers bearing lymph follicles and lymphoid cells in the medullary cord, at early stages of metastasis. These cytokines returned to basal levels 48 hours after inoculation. In vitro studies have demonstrated that IL-2 derived from CD4⁺ cells causes release of significant amounts of IFN-γ from CD8⁺ cells (14). However, in this study, the Th1 cytokines IFN-γ and IL-12 were not up-regulated at early stages of cancer cell migration, but their levels did decrease at later stages of metastasis, when the cancer cells began to proliferate. The profile of the Th2 cytokine IL-4 was similar to that of IFN-γ and IL-12. Clearly, the lymph nodes are showing immunologic unresponsiveness on initial cancer cell invasion and immunologic exhaustion at later periods of metastasis.

The antitumor effect is the result of cooperation between T cells and macrophages (34), and it has been reported that in vivo inhibition of macrophage function abrogates the therapeutic efficacy of adoptively transferred lymphoid cells in animal models (35, 36). In the present study, depletion of lymph node macrophages abrogated transient increases in TNF-α and IL-1β. This result indicates that TNF-α and IL-1β induced at early stages of metastasis were derived from lymph node macrophages. Induction of TNF-α and IL-1β possibly enhanced secretion of IL-2 from lymph node T cells transiently, but this propagation of cytokines was attenuated by macrophage depletion. The immunohistochemical analysis demonstrated that TNF-α and IL-1β were present in different macrophage populations. After contacting migrant cancer cells in the marginal sinus, ED3⁺ macrophages beneath the sinus showed a transient increase in TNF-α expression. Subsequently, medullary sinus macrophages, which are located downstream of the marginal sinus, secreted IL-1β. On initial entry of cancer cells into the marginal sinus, certain signals may be sent to parasinus macrophages, and MIF released from cancer cells is one of the candidates for mediation of up-regulation of TNF-α and IL-1β in the macrophages because MIF has been found to override glucocorticoid-mediated suppression of TNF-α and IL-1β secretion.
(37). Activation of macrophages is also produced by cytokines released by stimulated lymphocytes, and IFN-γ has been shown to activate macrophages to become tumoricidal (38). However, in this study, IFN-γ in the regional lymph node did not increase after cancer cell inoculation or NAAI pretreatment for macrophage activation, and an inadequate IFN-γ response is also found in the sentinel lymph node of melanoma-bearing patients (39). IL-12 has been found to enhance IFN-γ production and activate T cells, resulting in tumor growth inhibition (40), but in the present study, IL-12 did not up-regulate IFN-γ after cancer cell inoculation. Activation of macrophages by NAAI pretreatment suppressed lymph node metastasis in this study but did not alter the cytokine profiles after cancer cell inoculation. It is possible that NAAI elicited the tumoricidal effect of macrophages through mechanisms other than cytokine induction, such as enhancement of the O2-generating system (41) or production of prostaglandins and collagenase (42). Propagation of the cytokine network and cooperation between lymphocytes and macrophages for anti-tumor immunity did not function properly in the regional lymph nodes, and this may be related to the limited anti-metastatic capability of these lymph nodes. Furthermore, it is possible that the release of unknown immunosuppressive factors from cancer cells may induce a localized and specific paralysis of cytokine responses in the regional lymph nodes.

In conclusion, in vivo visualization of cancer cell migration in the regional lymph node confirmed that the marginal sinus constitutes a mechanical barrier against passage of cancer cells. Macrophages beneath the marginal sinus form the first line of defense against cancer cell invasion. After an initial increase, the number of cancer cells in the marginal sinus declined until 48 hours after inoculation, probably due to migration of cancer cells or cancer-cell-incorporating macrophages to the medulla. Immune responses occurring include germinal center activation, followed by lymphoid cell proliferation and sinus histiocytosis, which are consistent with histologic manifestations of regional lymph nodes in cancer patients with improved survival. Thus, lymph node do have the capability to eliminate cancer cells temporarily. However, these immune responses diminished after 4 days of inoculation, and then the cancer invaded the node and completed the metastasis process. The temporary induction of the cytokines TNF-α and IL-1β in parasinus macrophages occurred at early stages of metastasis. The depletion of immunocompetent cells due to unresponsiveness and exhaustion of lymph node cytokines may then be responsible for the subsequent growth of cancer cells in the regional lymph node.

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