B16-BL6 melanoma cells release inhibitory factor(s) of active pump activity in isolated lymph vessels

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Nakaya, Kei, Risuke Mizuno, and Toshio Ohhashi. B16-BL6 melanoma cells release inhibitory factor(s) of active pump activity in isolated lymph vessels. Am J Physiol Cell Physiol 281: C1812–C1818, 2001.—We investigated whether supernatant cultured with melanoma cell lines B16-BL6 and K1735 or the Lewis lung carcinoma cell line (LLC) can regulate lymphatic pump activity with bioassay preparations isolated from murine iliac lymph vessels. B16-BL6 and LLC supernatants caused significant dilation of lymph microvessels with cessation of pump activity. B16-BL6 supernatant produced dose-related cessation of lymphatic pump activity. There was no significant tachyphylaxis in the supernatant-mediated inhibitory response of lymphatic pump activity. Pretreatment with 3 × 10–3 M N-nitro-L-arginine methyl ester (L-NAME) or 10–5 M or 10–6 M glibenclamide and 5 × 10–4 M 5-hydroxydecanoic acid caused significant reduction of supernatant-mediated inhibitory responses. Simultaneous treatment with 10–3 M L-arginine and 3 × 10–5 M L-NAME significantly lessened L-NAME-induced inhibition of the supernatant-mediated response, suggesting that endogenous nitric oxide (NO) plays important role in supernatant-mediated inhibitory responses. Chemical treatment dialyzed substances of <1,000 molecular weight (MW), producing complete reduction of the supernatant-mediated response. In contrast, pretreatment with heating or digestion with protease had no significant effect on supernatant-mediated response. These findings suggest that B16-BL6 cells may release nonpeptide substance(s) of <1,000 MW, resulting in significant cessation of lymphatic pump activity via production and release of endogenous NO and activation of mitochondrial ATP-sensitive K+ channels.

malignant melanoma; active lymph transport; nitric oxide; mitochondrial adenosine 5′-triphosphate-sensitive potassium channel

THERE ARE CERTAIN PATTERNS of melanoma metastasis. Thus the initial site of distant metastasis is most commonly skin, subcutaneous tissues, liver, or lung (2, 3). During metastasis, melanoma cells can also easily penetrate lymph capillaries and spread through the lymphatic system. Tumor emboli may be trapped in the first drainage lymph node, or they may bypass those regional lymph nodes to form distant nodal metastasis (skip metastasis) (4, 13). However, the important clinical question of why melanoma cells can spread more quickly via the hematogenous route still remains unresolved.

The transport of lymph depends on passive and active driving forces as well as on the rate of lymph production in organs and tissues (14). The active driving mechanism plays a pivotal role in the centripetal propulsion of lymph, which is caused by the intrinsic active pump activity of the lymph vessels (6, 14). The rhythm and amplitude of the active pump activity are modified by neural and mechanical factors as well as humoral factors (7, 9, 15).

Little information exists, however, regarding potential effects of chemical substances released from melanoma cells on the active lymph transport mechanisms of lymph vessels. Thus, to understand the factors governing the spread of melanoma cells through the lymphatic system, we examined whether the malignant melanoma cell lines B16-BL6 and K1735 can release chemical substance(s) that modifies intrinsic active pump activity of lymph vessels isolated for bioassay and then investigated physiologically or pharmacologically the chemical properties of the substance(s).

MATERIALS AND METHODS

Five-week-old male ddY mice (body wt ~25 g, n = 62) were used for the present studies. The mice were housed in an environmentally controlled vivarium and fed a standard pellet diet and water ad libitum. All experimental protocols were approved by the Animal Ethics Committee, Shinshu University School of Medicine, in accordance with the principles and guidelines of the American Council on Animal Care.

Cell culture. B16-BL6 murine melanoma cells, transformed 3Y1 (SR-3Y1-2) cells, and Lewis murine lung carcinoma cells (LLC) were kindly provided by Dr. S. Taniguchi (Shinshu University School of Medicine). K1735 M2 murine melanoma cells were donated by Dr. I. J. Fidler (MD Anderson Cancer Center, University of Texas). In brief, a transformed rat fibroblastic cell line, SR-3Y1-2, was established from an embryonic cell line 3Y1-B clone1–6 after being infected with a Raus sarcoma virus (21). These cells were seeded into 100-mm tissue culture dishes (Corning) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; 10.220.33.5 on August 27, 2017 http://ajpcell.physiology.org/ Downloaded from

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Dainihonseiyaku) supplemented with 10% fetal bovine serum (JHR Bioscience), penicillin (100 U/ml; Sanko Junyaku, Tokyo, Japan), streptomycin (100 μg/ml; Sanko Junyaku), and amphotericin B (250 μg/l; Sigma, St. Louis, MO). The cells were grown at 37°C in a humidified incubator with 5% CO₂-95% air. The culture medium was changed every 2 or 3 days. The same number of the cells (1,000 cells/ml) were cultured to a postconfluent condition in the dishes, and then the cells were rinsed twice with conditioned phosphate buffer solution (Hanks’ buffered salt solution, pH 7.4; Sanko Junyaku). Forty-eight hours after resuspension in 15 ml of nutrient mixture [Ham's F-12 (GIBCO BRL-Life Technologies)-DMEM (1:1) mixture medium without serum], a uniform volume of supernatant (15 ml/dish) was collected from the seven culture dishes and then centrifuged (1,000 rpm for 5 min) and filtered (φ = 0.2 μm, Millipore) to remove cell debris. Ten milliliters of supernatant [from 105 ml (15 ml × 7 dishes)] was then diluted at a concentration of 20% with Krebs-bicarbonate solution (in mM: 120.0 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄, 5.5 glucose, and 25.0 NaHCO₃). This neutralized the concentration of chemical substances in the medium. In some experiments, B16-BL6 supernatant solutions at different concentrations (10.0%, 20.0%, 40.0%, and 100%) were constructed with appropriate volumes of Krebs-bicarbonate solution.

**Lymphatic bioassay preparation.** The mice were anesthetized with pentobarbital sodium (50 mg/kg ip). After an abdominal incision, iliac lymph microvessels with their lymph nodes were excised and placed in a petri dish containing cold (4°C) Krebs-bicarbonate solution. With the use of microsurgical instruments and an operating microscope, the lymph microvessels (n = 62; 101–244 μm in maximum diameter, 3 mm in length) were isolated and then transferred to a 10-ml organ chamber with two glass micropipettes containing Krebs-bicarbonate solution. After each lymph microvessel was mounted on a pipette (proximal) and secured with several sutures, the perfusion pressure was raised to 4 cmH₂O to flush out and clear the vessel. The distal end of the vessel was then mounted onto the outflow micropipette (distal). The proximal and distal micropipettes were connected with Tygon tubing with a custom-made diameter detection device using an edge-detection method (16). These changes were recorded on both a video-cassette recorder (Toshiba) and a direct-writing oscillograph (Sanei-Sokki Recti 8K). The intraluminal pressure in the microvessels was kept at 3–4 cmH₂O by elevating a 50-ml syringe connected to the inflow tubing while the outflow tubing was closed with a stopcock throughout the experiments. This pressure was optimal for production of active pump activity of the isolated murine lymph microvessels (10, 12).

**Experimental protocol.** To evaluate functional viability of endothelial cells, 10⁻² M acetylcholine (ACh) was first perfused extraluminally over all of the lymph microvessels before the experiments started. In the first experimental protocol, the effects of B16-BL6 supernatant, transformed 3Y1 supernatant, K1735 supernatant, LLC supernatant, or vehicle (culture medium without any cells) on active pump activity of the isolated lymph microvessels were evaluated by 3-min extraluminal perfusion of Krebs-bicarbonate solution containing the test supernatant or vehicle.

In the second protocol, the B16-BL6 supernatant-induced responses of the isolated lymph microvessels were examined in the presence or absence of glibenclamide (a selective ATP-sensitive K⁺ channel blocker; 10⁻⁷ and 10⁻⁶ M), iberiotoxin (IBTX, Ca²⁺-activated K⁺ channel blocker; 3 × 10⁻⁴ M), 5-hydroxydecanoic acid (5-HD, a selective mitochondrial ATP-sensitive K⁺ channel blocker; 5 × 10⁻⁴ M), NO-nitro-l-arginine methyl ester (l-NAME, an inhibitor of endogenous nitric oxide; 3 × 10⁻⁵ M), l-arginine (a substrate of endogenous nitric oxide; 10⁻⁵ M), and indomethacin (an inhibitor of cyclooxygenase; 10⁻⁵ M). The lymph microvessels were pre-treated with the various blockers for 30 min before extraluminal perfusion of B16-BL6 supernatant into the organ chamber.

In the third protocol, to evaluate chemical properties of the substances released from B16-BL6 melanoma cells, the effects of chemically or physically modified supernatants on lymphatic pump activity were studied. The modified supernatants were produced as follows. The supernatant was first boiled at 100°C for 30 min. Next, the supernatant was treated with protease (pronase E, 1 μg/ml; Sigma) at 37°C overnight, the reaction of which was terminated by heating at 80°C for 30 min. Finally, the supernatant was dialyzed by using a tubing of dialysis membrane (mol wt 1,000; Spectrum Medical Industries). The tubing was put into a buffer medium [F-12-DMEM (1:1) medium] for dialysis at 4°C overnight. The supernatant trapped inside the membrane was then used for the bioassay. Thus the supernatant contained no chemical substance <1,000 in molecular weight.

**Drugs.** Salts (Wako), ACh chloride (Daiichiseiyaku, Tokyo, Japan), glibenclamide (RBI), IBTX (Peptide Institute, Osaka, Japan), 5-HD, l-NAME, l-arginine, and indomethacin (Sigma) were used in the present study. Glibenclamide and indomethacin were diluted with dimethyl sulfoxide (DMSO) and ethanol, respectively. The concentrations of DMSO and ethanol did not exceed 0.036% in the organ chamber; these concentrations did not affect the active pump activity of the isolated lymph microvessels. Concentrations of drugs are expressed as the final concentration in the organ chamber. All salts and drugs were prepared on the day of the experiment.

**Statistical analyses.** The supernatant-induced inhibitory response is expressed as a percentage of inhibition of active pump activity of the isolated lymph microvessels. Thus the averaged frequency (times/min) of the pump activity during the occurrence of supernatant-induced response was normalized by that obtained before the application of supernatant.

The data are presented as means ± SE, and n indicates the number of vessels. Significant differences (P < 0.05) were determined by one-way analysis of variance (ANOVA), followed by Duncan’s post hoc test and paired Student’s t-test, as appropriate.

**RESULTS**

Isolated bioassay lymph microvessels of mice exhibited regular active pump activity at an intraluminal...
pressure of 3–4 cmH₂O. The maximum and minimum diameters of lymph microvessels were calculated to be 177.7 ± 6.5 (n = 62) and 159.2 ± 5.3 (n = 62) μm, respectively. The frequency of the pump activity of microvessels was 13.8 ± 0.2 min⁻¹ (n = 62).

Effects of B16-BL6 supernatant on lymphatic pump activity. Figure 1 shows representative tracings of the effects of B16-BL6, transformed 3Y1, LLC, and K1735 supernatants diluted by 20% and the culture medium itself (vehicle) on the active pump activity of an isolated lymph microvessel. B16-BL6 and LLC supernatants caused a significant dilation of the lymph microvessel with cessation of pump activity (Fig. 1, A and D). In contrast, both transformed 3Y1 supernatant and vehicle had no significant effect on lymphatic active pump activity (Fig. 1, B and C). In 11 of 15 lymph microvessels, K1735 supernatant also produced no significant effect on lymphatic pump activity (Fig. 1E). No tachyphylaxis was observed in the B16-BL6 supernatant-induced inhibitory response of the lymphatic pump activity. Repeated administrations (3 times) of the B16-BL6 supernatant into the same bioassay lymph microvessel at 30-min intervals produced a marked inhibition of the pump activity in a similar manner. The inhibitory responses of lymphatic pump activity induced by the first, second, and third administrations of B16-BL6 supernatant were −30.8 ± 0.3% (n = 3), −35.7 ± 7.9% (n = 3), and −35.7 ± 0.9% (n = 3), respectively.

B16-BL6 supernatant-mediated inhibitory responses of lymphatic pump activity were also demonstrated in a dose-dependent manner. Figure 2 shows representative recordings of dose-dependent inhibitory responses of lymphatic pump activity to B16-BL6 supernatant in the same lymph microvessel. The supernatant diluted by 10.0% with Krebs-bicarbonate solution caused no significant inhibitory effect on lymphatic pump activity (Fig. 2A). However, the supernatant diluted by >20.0% produced marked inhibition of active pump activity (Fig. 2, B–D). Thus the higher the ratio of dilation, the longer the B16-BL6 supernatant-induced cessation period of active lymphatic pump activity tends to be (Fig. 2, B–D).

Experimental data for B16-BL6 supernatant, transformed 3Y1 supernatant, and vehicle are summarized in Fig. 3A. The B16-BL6 supernatant-induced inhibition of the lymphatic pump activity was −41.1 ± 9.2% (n = 4; minus sign indicates inhibitory response), significantly less than that produced by transformed 3Y1 supernatant (+3.8 ± 2.8%, n = 4;
plus sign indicates accelerated response) or vehicle
(+4.7 ± 4.0%, n = 4).

Effects of K\(^+\) channel blockers on B16-BL6 supernatant-mediated inhibitory response of lymphatic pump activity. Figure 3B shows the summarized data for the effects of glibenclamide (10\(^{-7}\) M and 10\(^{-6}\) M) on B16-BL6 supernatant-mediated inhibitory responses of lymphatic pump activity. Pretreatment with glibenclamide caused significant dose-related reduction of B16-BL6 supernatant-mediated inhibitory response [control, −51.1 ± 3.5% (n = 5); 10\(^{-7}\) M glibenclamide, −11.9 ± 2.5% (n = 5; P < 0.05 vs. control); 10\(^{-6}\) M glibenclamide, 7.0 ± 9.4% (n = 5; P < 0.05 vs. control)].

Figure 3C presents the summarized data for the effect of 5-HD (5 × 10\(^{-4}\) M) on B16-BL6 supernatant-mediated inhibitory responses of lymphatic pump activity. Pretreatment with 5-HD produced a significant reduction of the inhibitory response [control, −45.8 ± 2.5% (n = 4); 5 × 10\(^{-4}\) M 5-HD, −6.4 ± 5.8% (n = 4; P < 0.05 vs. control)].

Figure 3D shows the summarized data for the effect of IBTX (3 × 10\(^{-8}\) M) on B16-BL6 supernatant-mediated inhibitory responses of lymphatic pump activity. Pretreatment with IBTX had no significant effect on the frequency of active lymph pump activity.

Effects of L-NAME, L-NAME and L-arginine, or indomethacin on B16-BL6 supernatant-mediated inhibitory response of lymphatic pump activity. The B16-BL6 supernatant-mediated inhibitory response was significantly reduced by pretreatment with 3 × 10\(^{-5}\) M L-NAME. This L-NAME-mediated inhibitory effect was significantly lessened by simultaneous treatment with 3 × 10\(^{-5}\) M L-NAME + 10\(^{-3}\) M L-arginine (E) and 10\(^{-5}\) M indomethacin (F) on the B16-BL6 supernatant-mediated inhibitory response of lymphatic pump activity. The abscissas are as in A. *Significantly different (P < 0.05) vs. control; †significantly different (P < 0.05) vs. pretreatment with L-NAME.
l-NAME, in the presence of l-NAME (3 × 10^{-5} M) alone, and in the presence of l-NAME (3 × 10^{-5} M) + l-arginine (10^{-5} M) were −53.7 ± 13.2% (n = 4), −7.7 ± 7.4% (n = 4; P < 0.05 vs. control), and −27.2 ± 9.8% (n = 4; P < 0.05 vs. l-NAME alone), respectively.

Pretreatment with 1-NAME (3 × 10^{-5} M) itself significantly constricted the maximal and minimal diameters and increased the frequency of lymphatic pump activity. Additional treatment with 10^{-3} M l-arginine and 3 × 10^{-5} M l-NAME also significantly lessened the l-NAME-induced constriction of the lymph vessel and increased the frequency of active pump activity.

In contrast, pretreatment with 10^{-5} M indomethacin had no significant effect on the B16-BL6 supernatant-mediated inhibitory response. These data are summarized in Fig. 3F [control, −44.8 ± 6.3% (n = 4); 10^{-5} M indomethacin −49.9 ± 11.1% (n = 4; not significant)]. Pretreatment with 10^{-5} M indomethacin itself produced no significant effect on lymphatic pump activity [maximal diameter: control, 168.8 ± 21.4 μm (n = 4) and 10^{-5} M indomethacin, 167.0 ± 23.1 μm (n = 4; not significant); frequency: control, 14.2 ± 0.8 min^{-1} (n = 4) and 10^{-5} M indomethacin, 15.1 ± 0.5 min^{-1} (n = 4; not significant)].

Effects of heating, enzymatic digestion with protease, or dialysis of B16-BL6 supernatant on supernatant-mediated inhibitory response of lymphatic pump activity. Pretreatment with heating or enzymatic digestion of the B16-BL6 supernatant with protease had no significant effect on the B16-BL6 supernatant-mediated inhibitory response of lymphatic pump activity. Figure 4, A and B, shows these summarized data [heated, −54.2 ± 9.0% (n = 4) vs. control, −40.8 ± 11.5% (n = 4; not significant); protease, −43.1 ± 10.9% (n = 4) vs. control, −36.4 ± 7.4% (n = 4; not significant)]. In contrast, pretreatment with dialysis significantly reduced the B16-BL6 supernatant-mediated inhibitory response. Figure 4C shows the summarized data [control, −43.7 ± 14.9% (n = 4) vs. dialysis, +3.8 ± 2.4% (n = 4; P < 0.05)].

**DISCUSSION**

Release of inhibitory substance(s) on lymphatic pump activity by malignant melanoma cell line. The lymphatic system plays an important role in regulating the transport of extracellular fluids and macromolecular substances in tissues. Thus lymph vessels act to return fluid and protein that escape from the capillary blood vessels to the systemic circulation. In the process of the transport, the escaped fluid and protein enter into the initial microlymphatics by a transient pressure gradient between the interstitial space and the initial lymphatics (1, 18). To accomplish these tasks, larger collecting lymph vessels work as a series of lymphatic pumps (lymphatic pump activity) that propel the lymph fluid centripetally by rhythmic constriction and dilation.

Our major findings in this study are summarized as follows. B16-BL6 and LLC supernatants caused a significant dilation of isolated murine iliac lymph microvessels with cessation of lymphatic pump activity. The supernatant cultured with B16-BL6 malignant melanoma cells caused a dose-related reduction of lymphatic pump activity. The B16-BL6 supernatant-mediated inhibitory response was significantly reduced by pretreatment with glibenclamide, 5-HD, or l-NAME. An additional treatment of l-arginine with l-NAME significantly lessened the l-NAME-induced reduction of the B16-BL6 supernatant-mediated inhibitory response. The concentrations of glibenclamide (10^{-7} and 10^{-6} M), 5-HD (5 × 10^{-4} M), and l-NAME (3 × 10^{-5} M) used in the present experiments are well known to selectively block an ATP-sensitive K+ channel, a mitochondrial ATP-sensitive K+ channel, and production of endogenous nitric oxide, respectively (11, 12, 17). Pretreatment with 3 × 10^{-8} M IBTX or 10^{-5} M indomethacin had no significant effect on the B16-BL6 supernatant-mediated inhibitory response.
tant-mediated inhibitory response of lymph pump activity.

The chemical treatment dialyzed substances of <1,000 molecular weight, causing a complete reduction of the B16-BL6 supernatant-mediated inhibitory response of lymphatic pump activity. In contrast, pre-treatment with heating or enzymatic digestion of the B16-BL6 supernatant with protease produced no significant effect on the supernatant-mediated inhibitory response. These findings suggest that the malignant melanoma cell line B16-BL6 may release nonpeptide substance(s) of <1,000 molecular weight. This conclusion is strongly compatible with the other experimental findings that transformed 3Y1 cell supernatant diluted by 20.0% and the culture medium itself (as vehicle) had no significant effect on lymphatic pump activity and that no tachyphylaxis was observed in the B16-BL6 supernatant-mediated inhibitory response of lymphatic pump activity.

The findings also suggest that the nonpeptide substance(s) released from melanoma cells can simultaneously activate ATP-sensitive K+ channels of lymphatic smooth muscles and production and release of endogenous nitric oxide from the lymph vessels, which result in a marked dilation of the bioassay lymph vessels and a complete cessation of lymphatic pump activity. Activation of calcium-dependent K+ channels in lymphatic vessels does not contribute to the B16-BL6-mediated inhibitory response, because the concentrations are known to inhibit significantly the activities of calcium-dependent K+ channels and cyclooxygenase in tissues (8, 9, 11, 20). This conclusion may be strongly supported by several studies finding that endogenous nitric oxide released from lymphatic endothelial cells inhibits lymphatic active pump activity in vivo and in vitro (15, 19, 22) and that activity of ATP-sensitive K+ channels plays a pivotal role in the regulation of lymphatic pump activity (11). Thus lymphatic endothelium-derived nitric oxide causes a significant reduction of the rhythm and amplitude of intrinsic spontaneous contractions in bovine mesenteric lymphatics (22) and a relaxation of lymphatic smooth muscles in isolated dog thoracic ducts (15). Endogenous nitric oxide has been shown to physiologically modulate lymphatic pump activity in rat mesentery in vivo (19). Thus endogenous nitric oxide is one of the key substances in the control of active lymph transport mechanisms in vivo and in vitro.

In addition, activation of the ATP-sensitive K+ channels on the lymphatic smooth muscles is also known to produce cessation of the spontaneous contractions in rat isolated mesenteric lymph microvessels (11). ATP is also known to modulate lymphatic vasomotion through an activation of the endothelial P2Y receptor (5). It may be reasonable to hypothesize that a high concentration of the nonpeptide substance(s) released from cutaneous melanoma cells diffuses the interstitial space, penetrates into the lymph capillaries, and then modulates active lymph transport mechanisms. Thus these substances cause dilation of lymph vessels and reduction of the lymphatic pump activity, which may lead to decreased lymph flow and resulting edema of the tumor tissue. Microenvironmental edema in the tumor tissue may affect redistribution of tumor cells through the regional lymph microvessels, which may contribute, in part, to the occurrence of the skip metastasis of cutaneous melanoma. Further investigation will be needed to study the physiological and pathophysiological significance of the edema formation in tumor tissues for governing the spread of carcinoma cells.

Another important aspect of the present study is that murine LLC supernatant can produce a marked dilation of the same isolated lymph microvessels with a complete cessation of the lymphatic pump activity (Fig. 1D), the responses of which are quite similar to those induced by the B16-BL6 supernatant. In contrast, in 11 of 15 lymph microvessels K1735 melanoma cell supernatant caused no significant effect on the lymphatic pump activity (Fig. 1E). Thus there is a marked heterogeneity between carcinoma cells in production and release of chemical substances that can modify lymphatic pump activity. There is, however, no evidence in the present study to explain the heterogeneity between the carcinoma cells. Further investigation will be also needed in the future to evaluate more exactly the chemical properties of the substances released from the carcinoma cells and to examine the relationships between the amount of the chemical substances released and broader implications for tumor metastasis.

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