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Original article

Aspirin reduces lung cancer metastasis to regional lymph nodes

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ABSTRACT

Background: Lung cancer is the main cause of cancer-related death worldwide. The high mortality is probably attributable to early metastasis; however, the mechanism underlying metastasis to regional lymph nodes is still unknown. Cyclooxygenase (COX)-derived prostaglandin E_2 (PGE_2) induces tumor growth and metastasis and is associated with a poor prognosis. The present study investigated the effect of an authentic COX inhibitor, aspirin, on regional lymph node metastasis during the development of lung cancer in mice.
Methods: An orthotopic intrapulmonary implantation model based on male C57BL/6 (6–8-weeks-old) mice was used. The lungs were injected with a solution containing Lewis lung carcinoma (LLC) cells overexpressing green fluorescent protein (GFP) and BD Matrigel®. The effect of aspirin on mediastinal lymph node metastasis of LCC cells from the primary injection sites was then examined.
Results: The implantation process took approximately 30 s per mouse and operative mortality was 10%. Single pulmonary nodules developed at the implanted site in 95% of animals, and regional mediastinal lymph node metastasis was observed at 14 days post-LLC-GFP cell injection in all mice that formed a primary lung tumor. The mean survival time of mice injected with LLC-GFP cells was 15 ± 3 days (range, 12–22 days). Histopathological analysis revealed that no metastatic tumors developed in the regional mediastinal lymph nodes by Day 10–12 post-LLC-GFP cell injection and no metastasis to distant organs or distant lymph nodes was observed by Day 21 post-injection. Oral administration of aspirin (100 mg/kg, twice a day) after LLC-GFP cell injection inhibited metastasis to the regional lymph nodes, with no significant suppression of primary tumor growth in the lungs. Aspirin treatment led to a significant reduction in mortality ($P < 0.0001$).
Conclusions: The present lymph node metastasis model is useful for evaluating the efficacy of agents that inhibit tumor metastasis to the regional lymph nodes. Aspirin reduced the metastasis of LLC-GFP cells injection to the regional lymph nodes, with a significant reduction in mortality. These findings suggested that COX inhibitors have potential for preventing lymph node metastasis.

1. Introduction

Lung cancer is the most common cause of cancer mortality worldwide and non-small-cell lung cancer (NSCLC) accounts for 75–85% of all diagnosed lung cancers. Despite progress in surgical techniques, chemotherapy, and radiotherapy, the 5-year survival rate for patients with lung cancer is only 16% [1]. It is estimated that 226,160 new cases will be diagnosed in 2012 and 160,340 will die from the disease [2]. More than half of patients (56%) with lung cancer have either advanced or metastatic disease at the time of diagnosis and, even with chemotherapy, the median survival is 1 year or less [3,4]. Tumor recurrence and metastasis are the major causes of treatment failure and death. Metastasis a complex multistep process during which cells must acquire several distinct properties: loss of cell-to-cell adhesion and increased invasiveness, intravasation, and increased survival and proliferation are all prerequisites for the establishment of distant macrometastases [5–7]. Despite progress in other areas of cancer therapeutics, the complexities of the process mean that cancer metastasis is still poorly understood.

Prostanoids, including prostaglandins (PGs), are generated from arachidonic acid via cyclooxygenase (COX) and specific PG synthases. These are the rate-limiting enzymes that regulate PG biosynthesis in various tissues [8–14]. COX-2, an isoform of COX, is

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expressed at sites of inflammation and malignancy. We reported previously that COX-2 and receptor signaling by prostaglandins play crucial roles in lymphangiogenesis during chronic inflammation, secondary lymphedema, and tumor development [8–14]. More precise understanding of the mechanisms underlying lymph node metastases, together with identification of the molecules involved, will improve the prognosis of lung cancer patients. Previous studies show that mice treated with COX inhibitors are resistant to the development of colorectal neoplasia, suggesting that COX inhibitors may be good therapeutic options [15]. The same may be true for humans; epidemiological studies show that non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, a classical and authentic COX inhibitor, reduces the risk of developing several types of cancer [16]. However, the precise role played by COX in regional lymph node metastasis is not clear.

Here, we examined regional lymph node metastasis in a mouse model of lung cancer development, and tested the effect of aspirin on regional lymph node metastasis from the primary lung tumor. We found that aspirin inhibited the metastasis of implanted lung cancer cells to regional lymph nodes, resulting in a significant reduction in mortality. These results suggest that aspirin may reduce metastasis of lung cancer cells to the regional lymph nodes and has the potential to extend the lives of lung cancer patients.

2. Materials and methods

2.1. Cell lines

LLC cells, originally isolated from C57Bl/6 mice, were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Gibco by Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco by Life Technologies, Grand Island, NY) in a humidified atmosphere containing 5% CO_2. LLC cells were purchased from Riken Brc Cell Bank (RBRV-RCB2638; Tsukuba, Japan).

2.2. Retroviral transfection of the GFP gene

Murine GPF cDNA was cloned into a deficient retroviral vector, pLEGFP (Clontech by Takara, Tokyo, Japan), and then transfected into PT67 cells (Clontech by Takara, Tokyo, Japan). The cells were then selected by culture with G418 (Roche, Basel, Schweiz). The resulting temporarily infectious recombinant GFP-containing virus was used to infect NIH/3T3 cells, which were then selected with G418 to evaluate the infectious titer. The titer was approximately 1×10^3 cfu/ml. LLC-GFP cells were infected with the temporarily infectious retroviruses and selected with G418. The selected cells were designed as LLC-GFP.

2.3. Animals

Male C57BL/6 mice (6–8-weeks-old; 20–25 g) were obtained from the CLEA Japan (Tokyo, Japan). The mice were maintained at constant humidity ($60 \pm 5\%$) and temperature (20 ± 1 °C) and a 12-hour light/dark cycle. All animals were provided food and water *ad libitum*. All experiments were performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

2.4. Intrapulmonary implantation

LLC-GFP cells (growing in log phase) were suspended in PBS (10 μl; final cell density, 5×10^4/ml) containing 10 μl of BD Matrigel® (Becton Dickinson Labware, MA, USA) to prevent air leakage and hemothorax when injected into the lung. The cells were injected into the left lung parenchyma of mice anesthetized with ether [17]. A small skin incision (approximately 5 mm in length) was made in the chest wall in the left lateral thoracic region. While observing the motion of the left lung through the pleura, a 31 gauge needle attached to a 0.3 ml insulin syringe was directly inserted through the intercostal space into the lung (to a depth of 2–3 mm). After injecting the LLC cells, the skin incision was closed with a surgical suture.

Mice were sacrificed at various time points after LLC-GFP cell injection, and the long and short diameters of the primary tumor masses were measured manually. The tumor volume was calculated using ImageJ software (NIH, MD, USA). Lungs harboring a primary tumor nodule and the mediastinal lymph nodes were excised for histological examination and fluorescence microscopy. For the experiment to evaluate the inhibitory effect of NSAIDs, mice were orally administered aspirin (100 mg/kg, twice a day; Sigma-Aldrich, STL, USA) from Day 0 after LLC-GFP injection. At the end of the experiment, the mice were sacrificed with ether and the lungs were resected, fixed with 4% paraformaldehyde (PFA) or 10% formalin. Control (sham operation) mice were injected with LLC cell culture medium, PBS, and BD Matrigel®.

2.5. Fluorescence microscopy

Mice were sacrificed as described above, and we observed the status of mediastinal lymph node metastasis examined under a fluorescence microscope (VHX-1000, KEYENCE, Osaka, Japan). The arbitrary units of fluorescence intensity were analyzed using VHX-1000 software (KEYENCE) and were compared between the vehicle-treated group and aspirin-treated group.

2.6. Immunohistochemistry

Lung tissues were excised and immediately fixed with 4% paraformaldehyde phosphate buffer solution (0.1 mol/L; pH 7.4). After fixation, the tissues were dehydrated in a graded series of ethanol solutions and then embedded in paraffin. Sections (4 mm thick) were cut and mounted on glass slides, deparaffinized with xylene, and placed in acetone at 4 °C. The sections were then blocked with 1% bovine serum albumin-PBS and incubated with Universal DAKO LSAB® + system-HRP (DAKO, North America, CA, USA) with DAB and Mayer's hematoxylin solution. Negative control staining was performed by replacing the primary antibodies with 1% bovine serum albumin/PBS. Images were captured using a BX51 microscope (Olympus, Japan).

2.7. Quantitative real-time PCR

Each sample of excised tissue was immediately immersed in RNAlater RNA stabilization Reagent (QIAGEN Japan, Tokyo, Japan) and homogenized for 60 seconds at 6000 rpm using a MagNALyser (Roche diagnostics Inc., Mannheim, Germany). Harvested cells were washed three times with PBS and homogenized using a QIA Shredder (QIAGEN Japan, Tokyo, Japan). Total RNA was extracted from the homogenized tissues and cells using the RNeasy Mini Kit (QIAGEN Japan, Tokyo, Japan) and single-stranded cDNA was generated from 1 μg of total RNA by reverse transcription using ReverTra Ace (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. Quantitative PCR amplification was performed using SYBRR Premix Ex Taq (Takara Bio Inc., Shiga, Japan). The real-time RT-PCR primers for GFP were designed using Primer 3 software (http://primer3.source forge.net) on the basis of data obtained from GenBank. The following primers were used for real-time RT-PCR: GAPDH forward, 5′-ACATCAAGAAGGTGGTGAAGC-3′, and reverse, 5′-AAGGTGGAAGAGTGGGAGTTG-3′; GFP sense, 5′-ACTACAACAGC-CACAACGTCT-3′ and antisense, 5′-GGTGT TCTGCTGGTAGTGGTC-3′; and COX-2 sense, 5′-TGGGTGTGAAGGGAAATAAGG-3′ and antisense, 5′-CATCATATTTGAGCCTTGGGG-3′ (Sigma-Aldrich).