Dose-effect relationship of celecoxib, a selective cyclooxygenase-2 inhibitor prevents lymphangiogenesis in a lewis lung carcinoma

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Abstract

COX-2 overexpression and lymphangiogenesis have been related to early-metastasis and poor prognosis in lung cancer. In this study, we used Lewis Lung Carcinoma cell lines were seeded in C57BL/6 mice left groin subcutaneous to establish homograft models. These models were randomly divided into four groups: control group, low-dose (30 mg.kg\textsuperscript{-1}.d\textsuperscript{-1}) group, medium-dose (90 mg.kg\textsuperscript{-1}.d\textsuperscript{-1}) group, and high dose (180 mg.kg\textsuperscript{-1}.d\textsuperscript{-1}) group. Then we observed the tumor-bearing survival statuses and tumor volume changes of the mice. Transplanted tumor tissues were collected after 42 days and we made a detection of COX-2, VEGF-C expression and lymphatic microvessel density by immunohistochemical staining method. Immunohistochemical staining showed that celecoxib with medium and high-dose lowered the expressions of COX-2, VEGF-C and lymphatic microvessel density (LMVD). And compared with the control group, there were significant differences (P<0.05). Though expressive levels decreased slightly, there were no significant differences between Low-dose group and control group (P>0.05). The degree of inhibition was dose-related. This study suggests that celecoxib inhibits the growth of Lewis lung tumor and lymph node metastasis by reducing expressions of COX-2 and VEGF-C and inhibiting lymphangiogenesis which is related to dosage. That provides certain experimental basis for drug development of anti-cancer lymph node metastasis in early stage and the patients’ prognosis improvement.

1. Introduction

Lung cancer is by far the most widespread cause of cancer deaths in the world and this high mortality is probably attributable to early metastasis. Adenocarcinoma has already surpassed squamous cell carcinoma as the most...
frequent type of lung cancer [1, 2] treatment of lung adenocarcinoma often fails because some patients already have metastatic disease at diagnosis. Because of the poor prognosis associated with such early acquisition of a metastatic phenotype, the regional lymph nodes draining primary tumors are generally the first and the most common sites of metastasis for many of the major human malignancies, lung cancer is no exception.

Non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenases (COXs) and suppress prostaglandin (PG) synthesis are widely used as anti-inflammatory, antipyretic and analgesic agents. Epidemiological studies have established that the long-term intake of NSAIDs reduces the risk of colorectal cancer [3]. The key enzyme involved in prostanoid synthesis from arachidonic acid is designated as COX [4]. Two forms of this enzyme exist in the mammalian body, constitutive COX-1 and inducible COX-2. COX-2 is responsible for many inflammatory processes and is up-regulated by various tumor promoters and growth factors. It is overexpressed in breast, head and neck, colon, pancreatic and lung cancers among other tumors [5-9]. In several previous studies, the prognostic significance of elevated COX-2 expression in primary lung adenocarcinomas was evaluated [10].

Selective inhibition of COX-2 as celecoxib is a drug that was designed to treat the signs and symptoms of adult arthritis [11]. Celecoxib inhibits the inflammatory COX-2 enzyme at therapeutic doses in humans that do not lower gastrointestinal prostaglandin levels associated with mucosal protection [12]. In recent years, some studies suggested that celecoxib may help prevent lung cancer [13], Koch, A et al. make a statement to experimental and clinical phase II trials have indicated that the addition of the COX-2 inhibitor celecoxib to palliative chemotherapy might increase survival time in patients with advanced NSCLC [14].Celecoxib has become a drug that chemopreventive and therapeutic activities toward lung cancer and other epithelial malignancies [15]. Celecoxib activity in several animal models has been associated with the decrease of new blood vessel production in tumors, a decrease in new vessel ornamentation and an increase in tumor cell apoptosis. Many studies confirm celecoxib of lung cancer significantly inhibited angiogenesis [16, 17], but the role of lymphangiogenesis relatively little research, and different doses of celecoxib lymphatic metastasis of lung cancer mechanism of action is more rarely reported .

In this study, we used Lewis Lung Carcinoma cells and C57BL/6 mice to establish the lung carcinoma animal model, and investigated the application of different doses of a selective COX-2 inhibitor as celecoxib on the growth, lymphangiogenesis in mice implanted with Lewis Lung Carcinoma cells.

2. Materials and Methods

2.1 Cell Culture

Lewis Lung Carcinoma cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% air in DMEM containing 10% heat-inactivated fetal bovine serum plus penicillin-streptomycin under sterile tissue culture conditions.

2.2 Celecoxib

Stock solutions of celecoxib (Pfizer pharmaceutical co., LTD) were made by dissolving the compound in DMSO, and then were stored at -20°C.

2.3 Mouse Model and Tumor Irradiation

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Chinese Association for Laboratory Animal Sciences. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Affiliated Hospital of Medical College Qingdao University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Male C57BL/6 mice, 4- to 5-weeks-old, (Hunan Slack King of Laboratory Animal Co.,
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Ltd. Hunan, China) were used in our study maintained in a specific pathogen-free grade animal room until 8–9 weeks of age and weighing 20–22 g. They were housed at 6–7 per cage. For each experiment, 1×10^6 cells from one of several different Lewis Lung Carcinoma cell lines were injected subcutaneously into the left hind limb of each mouse. A tumor bulb could be seen on the left hind limb 7 days after the tumor cells were injected. In total, there were 60 tumor-bearing mice in this experiment. Approximately 7 days after the mice were injected with tumor cells, we started the experiments. At that time, homograft tumors, which grew singly, were found in all the animals. Animals were housed individually in special filter cages to maintain aseptic conditions and to prevent mutual damaging of the cranial window. The animals were provided with sterile standard pellet food and water ad libitum. There was no difference in tumor volumes among the 60 mice. The mice were then divided into 4 groups of 15 each, and each group received a different agent. These agents included: 0.9% sodium chloride solution (control group); the different doses of celecoxib (celecoxib, 30.90, 180 mg . kg⁻¹.d⁻¹, n=15). These agents were administered by gavage once daily. Treatment was continued until termination of experiments on day 42 after tumor implantation. The mice were sacrificed. All tumors were resected, weighed, and then fixed in 4% phosphate-buffered formaldehyde.

2.4 General observations and tumor measurements

The general physical condition of the mice was observed every day, and each was weighed twice a week. To obtain the tumor growth curve, we used a vernier caliper to measure the tumor diameters, and mean values were calculated. Tumor volume was calculated as: tumor volume = 1/2ab²π (a > b) where a is the maximum diameter of the tumor and b is the maximum diameter perpendicular to a. The tumor inhibitory rate was calculated as: Tumor inhibitory rate = 1-(mean tumor weight of treated group/mean tumor weight of control group) × 100%.

2.5 Histology and immunohistochemistry (IHC)

All mice were sacrificed under anesthesia tumor tissue samples were fixed with 10% buffered formalin, and embedded in paraffin after routine dehydration. Consecutive 4-μm sections were cut from each block and were immunostained for COX-2, VEGF-C, podoplanin. The sections were deparaffinized in xylene, and rehydrated through graded alcohol and deionized water. For antigen retrieval, the sections were heated in microwave oven in 0.3% citrate buffer (pH 6.0) for 3×5 min and washed with phosphate-buffered saline (PBS) for 3×5 min. Endogenous peroxidase were inactivated by 30 min incubation in methanol containing 3% H₂O₂, followed by PBS wash for 3×5 min. To block non-specific biding sites, the sections were further treated with normal horse serum for 15 min. The slides were immunostained with antimouse COX-2(1:150) (Bo orson China), antimouse VEGF-C(1:200) (Bo orson China), podoplanin(1:200)(Abcam Biotechnology, Inc USA) at 4°C overnight. Slides were then washed three times in PBS and exposed to a biotinylated secondary antibody (Bo orson China) for 20 min, followed by treatment with streptavidin peroxidase (Bo orson China). For color development, the slides were stained with 3, 3′-diaminobenzidine (DAB, Bo orson China), then were counterstained with hematoxylin.

Podoplanin positive vessels, found mainly in the marginal portion, had relatively large lumens. First observed at low magnification to express the hot zone, and then count the number of lymphatic three horizons averaged (n1+n2+n3)/3 counting the specimen LMVD at 400 times magnification. The positive expression of COX-2, VEGF-C substances appear as brownish yellow fine particles mainly located in the cytoplasm of cancer cells. we used Image-Pro Plus Software (Media Cybernetics Inc., Rockville, USA) analysis optical density measured its size, than that is positive and integral optical density measured area: AIOD = IOD/μm².
2.6. Statistical analysis

All data were presented as mean ± standard deviation (SD). The results were compared by one-way analysis of variance (ANOVA). The Least Significant Difference (LSD) T-test was used to test for differences between the groups. All statistical calculations were performed with the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA). Differences were considered significant at P<0.05.

3. Results

3.1 Effect of different doses of Celecoxib on tumor growth and quality of tumor-bearing mice life

Lewis Lung Carcinoma cell is a well-established carcinoma cell line in mice. 1×10⁶ cells were enough to induce subcutaneous tumor masses. 60 tumor-bearing mice were successfully created and randomly divided into four groups. They were treated with different doses of celecoxib or control group. Early after tumor formation in mice body weight and activity did not change significantly. But with the growth of tumors, the mental status, eating habits, and behavioral characteristics of the mice worsened in the control group. Control group rapid tumor growth, including five mice homografts of skin ulceration, surface ulceration. Celecoxib intervention, high-dose, medium-dose group compared with the control group was significantly slower tumor growth, tumor smaller and more regular tumor, adhesions with the surrounding rare. Low-dose group, slightly slow tumor growth, compared with the control group did not change significantly.

The tumor formation time and tumor growth of all groups of mice were closely monitored after Lewis Lung Carcinoma administration and the tumor weight was recorded at the endpoint of experiment. As shown in Figure 1, after tumor from day 10 onwards, mean tumor volumes in the treatment groups were smaller than that in the control group, with the lowest tumor volume recorded in high dose group. On the last day, the mean tumor volume was 6488.59 ± 1606.14 mm³ in the control group, 3879.37 ±1085.04 mm³ in the low-dose group, 1149.94 ±670.45 mm³ in the mid-dose group, 839.53±272.77 mm³ in the high dose group. There was no statistically significant difference in tumor volume between the groups before treatment while the tumor volume was remarkably decreased after treatment (P<0.05). The therapeutic effect was most marked in the high dose group (P<0.05).

Fig.1 The growth curves of tumor homografts in C57BL/6 mice of four groups (high dose, mid-dose, low dose, control group). The capacity of tumorigenicity in high dose and mid-dose group was significantly reduced. High dose group and Mid-dose group ※P<0.05 compared with Low dose group and Control group.

The tumor inhibitory in Lewis Lung Carcinoma tumor cells in the control group and the groups treated with different doses of celecoxib was assessed by the tumor inhibitory rate and tumor mass. As shown in Table 1, when mice were treated with different doses of celecoxib, the tumor inhibitory rate was obviously higher than that in the non treated mice (P<0.05). However, the high, medium dose was not significantly different between the two groups (P>0.05).
<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Tumor mass (g)</th>
<th>Tumor Inhibition rate(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>3.47±0.42</td>
<td></td>
</tr>
<tr>
<td>Celecoxib(mg.kg(^{-1}.d^{-1}))</td>
<td>15</td>
<td>2.92±0.43</td>
<td>13.3</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>15</td>
<td>1.88±0.32**</td>
<td>46.8</td>
</tr>
<tr>
<td>180</td>
<td>15</td>
<td>1.56±0.38**</td>
<td>56.3</td>
</tr>
</tbody>
</table>

Study group: Low-dose; Middle dose; High dose. Control group: tumor mass: **P= 0.0000 < 0.01, vs Control group. Low-dose group vs Control group: P= 0.9799 > 0.05.

3.2 Effect of different doses of Celecoxib on Lymphangiogenesis

We next analyzed whether celecoxib affect lymphangiogenesis. Immunohistochemistry results showed that lymphangiogenesis within tumor tissues was estimated in terms of LMVD on the section stained with anti-mouse podoplanin antibody. Podoplanin was mainly expressed in interstitial vascular endothelial cells around cancer nests, and positive reactions were occasionally seen in tumor cells, with relatively weak staining. Tumors of the mice treated with control or low dose celecoxib showed larger LMVD than the other two groups (P<0.05). However, there was no difference between the high dose and mid-dose group (P>0.05). The result is illustrated in Figure 2 and Table 2.

3.3 Effect of different doses of Celecoxib on COX-2, VEGF-C expression

COX-2, VEGF-C positive substances appear as brownish yellow fine particles mainly located in the cytoplasm of cancer cells. In addition, it was expressed in various degrees on cell membrane. The correlation with treatment factor was analyzed. Expression of COX-2, VEGF-C in mice treated with control or low dose celecoxib was significantly higher than other groups (P<0.05). However, no difference between the groups treated with the high dose and mid-dose group (P>0.05). The result is illustrated in Figure 3, Figure 4 and Table 2.
in Lewis Lung Carcinoma homografts among four groups were detected by immunohistochemistry. The arrows represent the particles positively expressed in tumor homografts. A: High dose group(180 mg.kg-1.d-1); B: Middle dose group(90 mg.kg-1.d-1); C: Low-dose group(30 mg.kg-1.d-1); D: Control group.

**Table 2** The expression of COX-2, VEGF-C protein and the LMVD value in different groups of Lewis Lung Carcinoma homografts. ($^x \pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>COX-2 protein</th>
<th>VEGF-C protein</th>
<th>LMVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.46±1.12</td>
<td>11.25±3.56</td>
<td>8.50±3.216</td>
</tr>
<tr>
<td>Low-dose</td>
<td>4.83±0.95</td>
<td>10.78±3.47</td>
<td>7.360±2.238</td>
</tr>
<tr>
<td>Middle dose*</td>
<td>2.35±0.65</td>
<td>7.78±2.23</td>
<td>4.177±2.344</td>
</tr>
<tr>
<td>High</td>
<td>2.26±0.43</td>
<td>6.69±1.87</td>
<td>3.445±1.355</td>
</tr>
</tbody>
</table>

*Study group: Low-dose; Middle dose; High dose. Control group: * P < 0.05, vs Control group; Low-dose vs Control group; Middle dose vs High dose: P > 0.05.

4. Discussion

The most recent epidemiological survey shows that lung adenocarcinoma has become the most common pathological type of non-small cell lung cancer (NSCLC) [18]. With the emergence of molecular targeted therapy in recent years, the survival time of some patients with lung adenocarcinoma prolongs obviously, and the quality of their life is also improved. However, clinical research [19] shows that the effective rate of tyrosine kinase inhibitors (TKIs) for epidermal growth factor receptor (EGFR) mutation-positive patients with lung adenocarcinoma can reach 70%-80%, while that for wild-type patients is only 10%-20%, and there are still many limitations in treatment. Even for patients to whom the treatment with EGFR-TKI is initially effective, with the extension of the treatment time, the acquired drug resistance will finally occur in almost all patients. Therefore, it has become a trend of current studies on lung cancer to explore targeted drugs which are widely applicable.

Cyclooxygenase, the key enzyme required for the conversion of arachidonic acid to prostaglandins, exists under two forms, known as COX-1 and COX-2. In recent years, lots of studies have proved that the overexpression of COX-2 as an inducible enzyme plays a crucial role in occurrence, development and poor prognosis of tumors [20,21], and that COX-2 is overexpressed in head and neck cancers, esophageal carcinomas, colon cancers, breast cancers, pancreatic cancers and prostate cancers. In 1998, Hida et al [22] first reported that high COX-2 expression was seen in approximately 70% of lung adenocarcinomas. The same study demonstrated a greater proportion of lung cancer cells staining positively with COX-2 in lymph node metastases compared to the corresponding primary tumor. High COX-2 expression can be...
observed in the whole process from precancerosis to cancer. Many other studies also have demonstrated the significance of COX-2 expression in lung cancer [23, 24]. It was found in recent studies that among lung adenocarcinomas, squamous cell carcinomas, large cell lung cancers and small cell lung cancers, COX-2 expression in adenocarcinomas was most significantly enhanced and its overexpression was seen in about approximately 70% of lung adenocarcinomas and most cases were in the progressive stage. COX-2 was almost not expressed in small cell lung cancers and expressed relatively weakly in squamous cell carcinomas. The positive rates of COX-2 expression in carcinomas in situ and invasive adenocarcinomas of the lung both exceeded 80% [25], indicating that COX-2 expression is already enhanced in the early stage of lung cancer and is mainly seen in NSCLC, especially in lung adenocarcinoma.

Celecoxib, as a new selective COX-2 inhibitor with a specific effect on COX-2, has no obvious impact on COX-1 expression in normal tissues and small untoward effect on digestive tract, kidney and blood, therefore has become a hot topic in the area of anti-cancer research. Through drawing the growth curves of transplanted tumors in each group, measuring the mass of tumors and calculating the tumor inhibition rates, it was found in the experiment that celecoxib had an inhibitory effect on the growth of the transplanted tumors in each administration group. The tumor inhibition effect was more significant in the middle and high-dose groups than in the low-dose group and there was no significant difference between the low-dose group and the control group (P > 0.05). It coincides with the studies of Masferrer et al [26] in which they found that celecoxib not only could reduce the growth of transplanted Lewis Lung Carcinoma with dose dependence, but also could inhibit lung metastasis of subcutaneously transplanted colon tumors in nude mice also with dose dependence. Presently, there are different points of view on the mechanism of celecoxib in inhabitation of the growth of tumor cells. Some scholars hold that selective COX-2 inhibitors inhibit the growth of tumors through inhibiting COX-2, reducing the generation of prostaglandin (PG) and suppressing the activity of multiple genes controlling cell growth and their expression products such as ERK2. Moreover, according to some other studies, celecoxib gives play to its tumor inhibition effect via COX-2-independent pathways [27, 28].

In recent years, studies on the anti-lung cancer effect of celecoxib mostly focus on its effect on lung cancer cells and angiogenesis, and only a few studies pay attention to its effect on lymphangiogenesis, especially, there are few reports on whether the effect of celecoxib is dose-dependent in protection against the genesis of lung cancer lymphatic vessels. As early as 2001, Jackson [29] proposed a theory about the genesis of tumor lymphatic microvessels. It was found that there were many factors involved in the genesis of lymphatic microvessels. At present, most scholars believe the genesis of tumor lymphatic microvessels is the key step in tumor growth and metastasis. The genesis of lymphatic microvessels, which plays an important role in facilitating lymphatic metastasis of early lung cancer, has become an independent adverse prognostic factor in NSCLC [30, 31]. Studies show that VECF-C expression can facilitate lymphangiogenesis; VEGF-C activates the specific receptor VEGFR-3 in the endothelium of lymphatic capillaries via a paracrine mode to stimulate receptor autophosphorylation, thus to facilitate the genesis of lymphatic microvessels and the metastasis of tumors. The findings of studies on tumors such as thyroid cancer, gastric cancer, colon cancer, esophagus cancer, head and neck squamous cell carcinoma and cervical cancer support the participation of VEGF-C/VEGFR-3 signaling pathway in tumor lymphangiogenesis or lymphangiectasia and the hyperplastic and dilated lymphatic vessels provide more opportunities for the contact between tumor cells and lymphatic endothelium and for invasion against lymphatic vessels and metastases to lymph nodes. In our experiment, through immunohistochemical staining,
demonstrated that COX-2 expression was reduced in tumor tissues under the effect of celecoxib and the corresponding VEGF-C expression and lymphangiogenesis were also obviously reduced, suggesting that COX-2 may have a synergistic or facilitating effect on VEGF-C expression and lymphangiogenesis. However, the specific mechanism is still debatable. Some scholars argue that the impact of COX-2 expression on VEGF-C changes involves multiple signaling pathways. COX-2 can catalyze the synthesis of prostaglandin 2 (PGE2), and the combination of PGE2 and prostaglandin receptor can activate this receptor. The activation of this receptor facilitates tyrosine phosphorylation of the human EGFR HER/Neu-2 with p38 mitogen activated protein kinase (MARK). This signal mediates and stimulates the transcription factor NF-Kb. Furthermore, it has been confirmed that signaling pathways including MAPK and NF-KB are all related to the regulation of VEGF-C expression in some tumor cells [32]. Some other studies suggest that COX-2 up-regulates VEGF-C expression via EP4 and EP1 receptor pathways to facilitate lymphangiogenesis, further to facilitate lymphatic metastasis [33, 34].

Podoplanin is a mucin-type transmembrane glycoprotein. Since it is expressed in lymphatic endothelial cells in the vascular system, lymphatic and blood vessels can be distinguished with the immunohistochemical method. The research findings of Schmid et al [35] show that lymphatic vessels and blood capillaries can be distinguished through double staining with CD34 and podoplanin. Cursiefen et al [36] conducted a study on corneal lymphangiogenesis with the adoption of immunogold labeling technique and immunoelectron microscopy. They found that the endothelial cells in which podoplanin were expressed had the ultrastructural features of lymphatic endothelial cells, therefore, podoplanin could be taken as a specific marker of lymphatic endothelial cells. Our experiment adopted immunohistochemical staining to detect the expression of podoplanin protein and count LMVD. The results show that the groups administrated with celecoxib have lower LMVD than the control group and dose dependence obviously exists in each administration group.

In summary, the present study found that different doses of celecoxib can inhibit the growth of Lewis lung tumor and lymphangiogenesis, reduce expressions of COX-2, VEGF-C. high, medium and low dose group dose group significantly inhibited, indicating that celecoxib may inhibit the expression of COX-2 in lung cancer, and reduced VEGF-C production, inhibition of lung cancer lymphangiogenesis, this inhibition in a dose-dependent manner. The results of this study will not only help to promote celecoxib as chemotherapy drugs in clinical practice, thereby improving chemotherapy to reduce the side effects of chemotherapy drugs, but also a way to study the anti-cancer lymph node metastasis in early stage micro and improve the prognosis of patients with lung cancer drug provides some experimental basis. Celecoxib inhibited lung lymphangiogenesis role in the existence of other mechanisms, mechanisms for more in-depth study on its ability to find a new target for the treatment of lung cancer has yet to be a large number of laboratory and clinical exploration, celecoxib applied to early clinical anti-cancer lymph node metastasis optimum safe dose has yet to be further explored.

5. Conclusions

Celecoxib inhibits the growth of Lewis lung tumor and lymph node metastasis by reducing expressions of COX-2 and VEGF-C and inhibiting lymphangiogenesis which is related to dosage. That provides certain experimental basis for drug development of anti-cancer lymph node metastasis in early stage and the patients prognosis improvement.

Competing interests

The authors declare that they have no competing interests.
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