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The Inhibitory Effect of Different Doses of Celecoxib on Lymphatic Microvessel Density in Tumor Homografts in C57BL/6 Mice*

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ABSTRACT Objective: To observe the effects of different doses of celecoxib on the growth of tumor homografts in C57BL/6 mice, COX-2 expression and lymphatic microvessel density and to explore the possible mechanism of action and dose-effect relationship of celecoxib on lymphangiogenesis of Lewis lung tumor. **Methods:** The cell lines of Lewis lung carcinoma were inoculated in the left inguinal subcutis of C57BL/6 mice for establishment of the tumor homograft model. They were randomly divided into four groups, including the control group, low-dose, medium-dose and high-dose celecoxib groups. The survival status and tumor volume change were observed in tumor-bearing mice. The mice were sacrificed 42 days after transplantation of tumors. The tumor tissue was collected for western blot semi-quantitative detection of COX-2 expression and lymphatic microvessel density (LMVD). **Results:** Semi-quantitative western blot determination showed that the expression levels of COX-2 and immunohistochemical-stained lymphatic microvessel density counting in the high- and medium-dose groups were significantly reduced, and the differences were statistically significant (P<0.05); the low-dose group was slightly reduced but not significantly different (P>0.05). The degree of inhibition was in a dose-dependent manner. **Conclusion:** The inhibition of celecoxib on the growth of Lewis lung cancer homografts and lymph node metastasis may be related to the down-regulation of COX-2 expression of lymphangiogenesis signaling pathways, and inhibition of lymphangiogenesis which is related to dosage.

Key words: Lymphangiogenesis; Celecoxib; Cyclooxygenase-2; LMVD; Western blot Chinese Library Classification(CLC): Q95-3; R734.2 Document code: A Article ID:1673-6273(2014)29-5629-04

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] and treatment of lung adenocarcinoma often fails because some patients already have metastatic disease at diagnosis. 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]. 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.

Selective inhibition of COX-2 as celecoxib is a drug that was designed to treat the signs and symptoms of adult arthritis^[10]. In recent years, some studies suggested that celecoxib may help prevent lung cancer, 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^[11,12]. Many studies confirm celecoxib of lung cancer significantly inhibited angiogenesis^[13,14], but the role of lymphangiogenes is relatively of 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 cell 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 cell.

1 Materials and Methods

1.1 Main reagents and materials

Lewis Lung Carcinoma cell line was purchased from Shanghai Institute of Cell Library. Celecoxib was purchased from Meilun Biotechnology Co.Ltd (Dalian,China). Rabbit anti-COX-2, a biotinylated secondary antibody, DAB were purchased from Bo orson Biotechnology Co.Ltd (Beijing,China). Antibodies against podoplanin and β-actin were from Abcam Biotechnology, Inc US-A. DMEM medium and fetal bovine serum (FBS) were purchased

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from Hyclone (USA).

1.2 Animals

Male C57BL/6 mice,4-to5-weeks-old, (Hunan Slack King of Laboratory Animal Co., Ltd. Hunan.China) were used in our study maintained in a specific pathogen-free grade animal room until 8-9weeks of age and weighing 20-22 g. They were housed at 6-7 per cage.

1.3 Cell Culture

Lewis Lung Carcinoma cell line was 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.

1.4 Mouse Model and Tumor Irradiation

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. 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: 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.

1.5 Detection of the COX-2 expression by Western Blot

Tumor tissues were prepared and Western blotting were performed according to previous procedures. Blocking and washing with phosphate-buffered saline, the polyvinylidene fluoride membranes were incubated overnight with rabbit anti-COX-2 (1: 200; dilution) as the primary antibodies. The secondary antibody used was horseradish-peroxidase-conjugated anti-IgG. Proteins were quantified using Image Acquisition and Analysis Systems.

1.6 Detection of MLVD by immunohistochemistry (IHC)

The slides were immunostained with Antimouse podoplanin (1:200; dilution) at 4 $^{\circ}$ C overnight. For color development, the slides were stained with3, 3'-diaminobenzidine (DAB), 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 (nl+n2+n3)/3 counting the specimen LMVD at 400 times magnification.

1.7 Statistical analysis

All data were presented as mean \pm 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.

2 Results

2.1 Effect of different doses of Celecoxib on Lymphangiogenesis

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. Statistical analysis showed that: the average number of micro-lymphatic Vision 400 times the control group, low dose, middle dose and higher dose group, was 8.503 ± 3.216 , 7.360 ± 2.238 , 4.177 ± 2.344 , 3.445 ± 1.355 . 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)(Fig.1).



Fig.1 LMVD (Lymphatic microvessel density) (DAB staining, × 400) in tumor homografts in C57BL/6 mice among four groups were detected by immunohistochemisty. 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

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2.2 Western blot analysis the expression of COX-2 protein

Western blot analysis was performed to determine the COX-2 protein expression in different treatment groups. Analysis revealed control group tumor tissues express higher levels of COX-2 protein than the high, medium dose of celecoxib treatment groups (P < 0.05). But no difference between the groups treated with the high dose or mid-dose of celecoxib (P > 0.05)(Fig.2).

3 Discussion

The latest data showed that lung adenocarcinoma had become the most common histological type of non-small cell lung cancer (NSCLC) ^[15]. With the recent emergence of molecular targeted therapy, the survival has been significantly prolonged in part of patients with lung cancer, and the quality of life has also been im-



Fig.2 Western blot analysis showing the levels of COX-2:a:Ratios of the surviving protein compared to the β-actin by different treatments. The ratios are expressed as Mean± SD, P< 0.05; b: Western blot shows the expression of COX-2 in each group

proved.

Cyclooxygenase is a key enzyme in the conversion from arachidonic acid to prostaglandins, in both COX-1 and COX-2 forms. In recent years, a large number of studies have confirmed the crucial role of overexpressed COX-2 in the occurrence, development and poor prognosis of tumors as an inducible enzyme. In 1998, Hida, et al ^[16] first reported high COX-2 expression in about 70% of lung cancer tissue, and the number of COX-2-positive cancer cells in lymph node metastases was higher than the primary tumors. High COX-2 expression existed in the whole process from precancerous lesions to cancer. A number of other studies have confirmed the importance of COX-2 expression in lung cancer. Recent studies found that high COX-2 overexpression existed in about 70% of patients with lung adenocarcinoma, mainly in progressive cases with a poor prognosis. The positive rates of COX-2 expression in the lung carcinoma in situ and invasive adenocarcinoma were over 80%^[17], indicating that increased COX-2 expression existed in early lung cancer and was mainly found in NSCLC.

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Celecoxib, as a new generation of selective COX-2 inhibitors. In recent years, the studies about the anti-cancer effect of celecoxib were mainly focused on its effect on cancer cells themselves and angiogenesis, but less involved in the lymphangiogenesis research, particularly less in whether the effect of celecoxib on anti-cancer lymphangiogenesis was a dose-dependent. As early as 2001, Jackson ^[18] proposed a theory of tumor lymphangiogenesis, and found that many factors had involved in lymphangiogenesis. Currently, most scholars believed that micro-lymphangiogenesis played an important promoting role in early lymphatic metastasis of lung cancer, which had been one of the independent poor prognostic factors of NSCLC. In this study, COX-2 expression was measured by semi-quantitative western blot determination and compared between different dose groups and the control group. The results showed that the expression levels of COX-2 were consistently decreased compared with the control group. The statistical analysis found that the expression levels of COX-2 in the highand medium-dose groups were significantly reduced compared with the control group, and the difference was statistically significant (P<0.05), but the low-dose group showed no significant difference compared with other groups (P>0.05). It was indicated that the inhibiting effect of celecoxib on COX-2 expression showed a dose-dependent manner. However, the specific mechanism was not very clear. Podoplanin was expressed on lymphatic endothelium. Since its expression was present in the lymphatic endothelial cells of vascular system, the lymphatic and blood vessels could be distinguished by immunohistochemistry methods. In this study, podoplanin expression and LMVD counting were detected in Lewis Lung Carcinoma homograft tissues in different groups by immunohistochemical staining. The results showed that LMVD in the high- and medium-dose groups was significantly reduced compared with the control group, and the difference was statistically significant (P<0.05), but the low-dose group showed no significant difference compared with other groups (P>0.05). Celecoxib might inhibit COX-2 expression, thereby inhibiting multiple signaling pathways of lymphangiogenesis, but the specific mechanism was not very clear. COX-2 might also increase VEGF-C expression to promote lymphangiogenesis via EP1 and EP4 receptor pathways, thereby promoting lymphatic metastasis^[19,20].

In conclusion, the present study found that different doses of celecoxib had inhibited the general survival state of Lewis Lung Carcinoma tumor-bearing mice, COX-2 expression and micro-lymphangiogenesis, especially in the high- and medium-dose groups, indicating that celecoxib might inhibit COX-2 expression in lung tissue, and block the lymphangiogenesis pathway, thus inhibiting lymphangiogenesis in lung cancer in a dose-dependent manner. The results of this study is not only conducive to promote celecoxib as chemotherapy drugs in clinical practice, thereby improving chemotherapy and reducing the side effects of chemotherapy drugs, but also provide some experimental basis for development of a new drug for anti-cancer lymph node metastasis in early lung cancer and improvement of the prognosis of patients. The optimum safe dose of celecoxib applied in early clinical anti-cancer lymph node metastasis is to be further explored.

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不同剂量的塞来昔布对 C57BL/6 小鼠移植瘤微淋巴管密度 抑制作用的研究 *

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摘要 目的:观察不同剂量的塞来昔布对 C57BL/6 小鼠肺癌移植瘤生长、COX-2 表达和微淋巴管密度影响,探讨塞来昔布对 C57BL/6 小鼠肺癌移植瘤淋巴管生成可能作用机制及量效关系。方法:将 Lewis 肺癌细胞株接种于 C57BL/6 小鼠左侧腹股沟皮 下建立移植瘤模型,随机分为4组:对照组、塞来昔布低剂量、中剂量、高剂量组。观察荷瘤小鼠生存状态,瘤体积变化,种瘤42 天 后牺牲小鼠,western blot 半定量检测 COX-2 表达及微淋巴管密度。结果:Western blot 半定量显示:塞来昔布高、中剂量组 COX-2 的表达水平及免疫组织化学染色微淋巴管密度计数均明显减低,差异有统计学意义(P<0.05),低剂量组略有减低但差异无统计学意义(P>0.05)。抑制程度呈明显的剂量依赖性。结论:塞来昔布抑制 Lewis 肺癌移植瘤的生长及淋巴转移,可能与下调 COX-2 的表达,阻遏了淋巴管生成的信号通路,抑制微淋巴管生成有关,该抑制作用呈一定的剂量相关性。 关键词:淋巴管的生成;塞来昔布;环氧化酶 -2;微淋巴管密度;免疫印迹分析

大健问:你口书的生成;圣术百冲;尔利化晦-2;佩你口书岔及;尤及中边分列

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