COX-2 increase tumor-associated angiogenesis and tumor growth by eNOS-dependent pathway

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eNOS 의존적 pathway를 통한 COX-2의 tumor 성장 증가와 tumor 혈관신생 증가

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Abstract

Cyclooxygenases (COX)-2 has been highly expressed in a variety of tumor cells and involved inflammatory process, tumor-associated angiogenesis, and vascular functions but the underlying mechanism is not clearly elucidated. We here investigated the molecular mechanism by which COX-2 regulates tumor-associated angiogenesis. *In vivo*, we injected B16-F1 cells overexpressed with COX-2 or mock in wild type or eNOS-deficient mice. Tumor cells overexpressed with COX-2 increase tumor-associated angiogenesis and tumor growth compared with control cells and that the effect of COX-2 was lower in eNOS-deficient mice than wild type mice. These results may contribute to further understanding of the regulation of angiogenesis by COX during tumor metastasis and inflammation.

1. Introduction

Angiogenesis is the formation of new blood vessels from pre-existing vessels, which enables the delivery of oxygen and nutrients and is strongly regulated in many physiological and pathological conditions. It is generally accepted that solid tumor growth and metastasis are dependent upon the acquisition of an adequate via new blood vessel formation [1, 2].

COX is the rate-limiting enzyme for the major production of prostaglandins from free arachidonic acid [3]. Two isoenzymes of COX have now been described. A constitutive enzyme COX-1 and inducible COX-2 to PGG/H_2, generating five primary bioactive PGs: PGE_2, $PGF_2\alpha$, PGI_2, and thromboxan A_2 by cell-specific isomerases and synthases [4-6]. COX-2 is highly expressed in a variety of tumor cells including breast, colorectal, colon, lung, prostate, esophageal and pancreatic carcinomas. Co-culture of endothelial cells with tumor cells promotes COX-dependent endothelial motility and assembly into capillary-like structure [7]. COX-2 overexpression is linked to all stages of tumor growth and metastasis through the formation of new blood vessels. This provides the strong evidence that COX-2 metabolites participates in angiogenic processes.

In the present study, we investigated the molecular mechanism and signaling pathway by which COX-2 regulates tumor-associated angiogenesis and tumor growth in vivo system. These results clearly show that eNOS-dependent NO production plays an important role in tumor angiogenesis and tumor growth by COX-2

expression.

2. Materials and Methods

2.1 Cell culture and transfection

Mouse melanoma cells (B16-F1) were cultured in DMEM medium maintained in 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human COX-2 expression vector (pcDNA3/COX-2) was transfected into the B16-F1 cells by Lipofectamine (Invitrogen, Carlsbad, CA), and clonal selection was performed by culturing the cells in medium containing 1.5 mg/ml of G418.

2.2 Western blotting

Two types of B16-F1 cells, control and COX-2 overexpressed cells were collected by scrapping and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid, 0.1% SDS). Cell lysates (50 μg protein) were electrophoresed on SDS-PAGE gel and transferred to PVDF membrane. Membrane were incubated with antibodies against COX-2 (New England Biolabs, Beverly, MA) and β-actin (Sigma, St. Louis, MO). After incubation, the corresponding secondary antibody signals were detected by the enhanced chemiluminescence reagents.

2.3 Tumor implantation

The mouse melanoma cell line B16-F1 cells stably transfected with empty vector (pcDNA3) and COX-2 gene (pcDNA3/COX-2) were grown in cell culture as described above. Cells were harvested and washed three times with PBS. The cell pellets were resuspended in PBS at a density of 4×10^6 cells/ml. The cell suspension (0.2 ml) was injected into the dorsal *s.c.* tissue of wild type and eNOS-deficient mice (6-8 weeks) following anesthesia. After 21 days, the tumors were excised with the encapsulating stromal tissues, dissected free fat and connective tissues, and weighed. The tumor volumes were determined by measuring the length (l) and width (w) and calculating the volume ($V = 1 \times w^2 \times 0.52$). Tumor tissues were homogenized in distilled water (four times the weight of the tumor tissue) in a Polytrone homogenizer. After centrifugation at 5000 x *g* for 30 min at 4℃, the hemoglobin concentration in the supernatant was measured using a Drabkin reagent kit 525 (Sigma, St. Louis, MO).

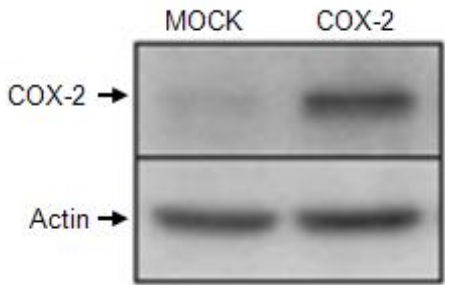
2.4 Statistical analysis

The data are presented as means standard deviation (S. D.) of at least three separate experiments in triplicate. Comparisons between two groups were analyzed using ANOVA, and significance was established at a p value 0.05.

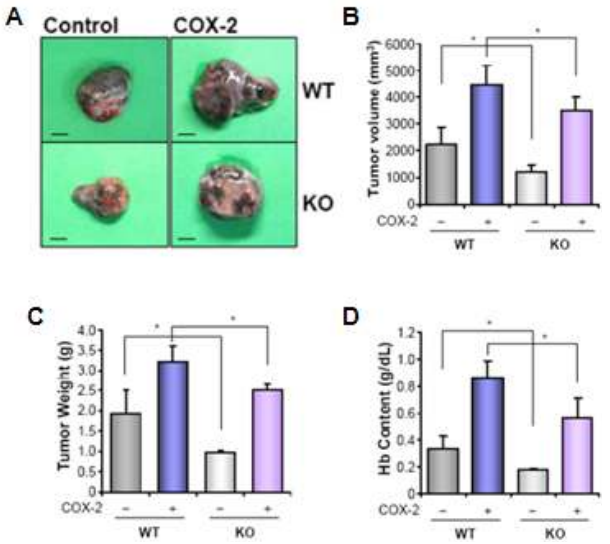
3. Results

In our pervious study, we showed that PGE_2, downstream of COX-2, plays a significant role in facilitating angiogenesis by activating the NO/cGMP signaling pathway throught PKA/PI3K/Akt-dependent increase in eNOS activity [8]. Furthermore, PGE_2 is a major product of COX and regulates tumor-associated angiogenesis and tumor growth [9-11]. To elucidate the significant role of the NO/cGMP pathway in COX-2-dependents *in vivo* angiogenesis and tumor growth, two types of B16-F1 cells, control and COX-2-overexpressed cells (Figure 1A), were inoculated into the dosal *s.c.* space of wild type and eNOS-deficient mice, and tumor-associated angiogenesis and tumor growth were determined at 21 days after inoculation. Tumor weight and volume were increased in both mice implanted with COX-2-overexpressed tumor cells compared with those in mice inoculated with control cells, and these increases were lower in eNOS-deficient mice than those in wild type mice (Figure 2 A, B and C). Since hemoglobin contents of tumor tissues are well correlated with histologically examined neovascularization, we measured the hemoglobin level as an indicator of

tumor-associated angiogenesis. Similarly, the hemoglobin contents were increased in the tumor tissues from both wild type and eNOS-deficient mice implanted with COX-2- overexpressed B16-F1 cells compared with those from control cell-implanted mice increments in the hemoglobin contents in eNOS-deficient mice, however, were significantly lower than those of wild type mice (Figure 2D). These results indicate that eNOS-dependent NO production plays as important role in tumor angiogenesis and tumor growth by COX-2 expression.



[Fig. 1] Western blot analyses of COX-2 levels in B16-F1 cells. B16-F1 tumor cells were transfed with vector alone or COX-2 expression vector by Lipofectamine, and clonal selection was performed by culturing the cells in medium containing 1.5 mg/ml of G418. COX-2 protein levels of these tumor cells were determined by Western blot analysis.



[Fig. 2] Cloned B16-F1 cells were inoculated into the dorsal s.c. space of wild type and eNOS-deficient mice. After 3 weeks, the tumor tissues were excised with the encapsulating stromal tissues and dissected free fat and connective tissues. Typical tumor appearance (A), tumor volume (B), and tumor weight (C), hemoglobin contents (D), were determined. Bar in (A) is 5 mm. Data shown in (A, B, and D) are the mean ± S.D. of 6 animals. $*P<0.05$.

4. Conclusion

The present study, we found that COX-2-overexpressed tumor cells increased tumor-associated angiogenesis and tumor growth *in vivo* compared with control cells and that these increases were significantly lowered in eNOS-deficient mice compared wild type animals. These findings indicate that eNOS-dependent NO production is involved in COX-2-dependent tumor-associated angiogenesis and tumor growth. These results suggested that the molecular mechanism for COX-2-dependent tumor growth and metastasis is associated with an increase in angiogenesis by the activation of the NO signaling pathway and that the NO production may be a potential therapeutic strategy for angiogenesis-associated several human diseases. In conclusion, we demonstrated that COX-2/NO pathway is a critically important factor for tumor growth and metastasis *in vivo*.

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