Title

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Antitumor effects of celecoxib in COX-2 expressing and non-expressing canine melanoma cell lines

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Abstract

Cyclooxygenase-2 (COX-2) is a potential target for chemoprevention and cancer therapy. Celecoxib, a selective COX-2 inhibitor, inhibits cell growth of various types of human cancer including malignant melanoma. In dogs, oral malignant melanoma represents the most common oral tumor and is often a fatal disease. Therefore, there is a desperate need to develop additional therapeutic strategies. The purpose of this study was to investigate the anticancer effects of celecoxib on canine malignant melanoma cell lines that express varying levels of COX-2. Celecoxib induced a significant anti-proliferative effect in both LMeC and CMeC-1 cells. In the CMeC cells, treatment of 50 μM celecoxib caused an increase in cells in the G0/G1 and a decreased proportion of cells in G-2 phase. In the LMeC cells, 50 μM of celecoxib led to an increase in the percentage of cells in the sub-G1 phase and a significant activation of caspase-3 when compared to CMeC-1 cells. In conclusion, these results demonstrate that celecoxib exhibits antitumor effects on canine melanoma LMeC and CMeC-1 cells by induction of G_1-S cell cycle arrest and apoptosis. Our data suggest that celecoxib might be effective as a chemotherapeutic agent against canine malignant melanoma.

Keywords

Antitumor effects; Canine melanoma; Celecoxib; COX-2 inhibitors

Melanomas are malignant tumors arising from melanocytes. Oral melanoma is the most common oral tumor in dogs (Goldschmidt, 1985). Unlike cutaneous melanomas of haired-skin, which are usually benign in the dog, oral melanoma is almost uniformly malignant and usually displays aggressive growth and metastasis to regional and distant sites (Ogilvie and

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Moore, 2006). Because of the lack of efficacious chemotherapeutic regimens for metastatic melanomas, several novel therapeutic strategies have been investigated (Rigel and Carucci, 2000; Withrow et al., 2012).

Cyclooxygenase-2 (COX-2) is expressed in more than half of spontaneous canine cancers and known as having a central role in the development and progression of some cancers (Pyrko et al., 2007). Increased COX-2 expression has been reported to mediate invasiveness of tumor cells (Kim et al., 2010), promotion of angiogenesis (Tegeder et al., 2001) and anti-apoptotic effects (Li et al., 2001). Altered COX-2 expression has been associated with the development and progression of human melanoma (Kuzbicki et al., 2006). In dogs, COX-2 was expressed in 21 of the 31 canine malignant melanomas (Pires et al., 2010), and oral malignant melanomas were specifically reported to have moderate to strong COX-2 expression.

Celecoxib (CELEBREX®, Onseral®, Pfizer, New York, USA), a selective COX-2 inhibitor, has been reported to inhibit the growth of human cancer cell lines (Bocca et al., 2011; Dhawan et al., 2010; Liu et al., 2009; North, 2001). There have been minimal reported data concerning the use of COX-2-specific inhibitors as potential antineoplastic drugs in canine malignant melanoma.

In this study, we investigated the anticancer effects of celecoxib either on COX-2 high expressing or null canine malignant melanoma cell lines.

Two different canine malignant melanoma cell lines (LMeC and CMeC-1) were used in our study (Inoue et al., 2004). Both cell lines were provided from Professor Nobuo Sasaki in the University of Tokyo. Description of detailed material and methods used in this investigation are provided as supplementary file.

COX-2 expression in CMeC-1 and LMeC cells treated with celecoxib was analyzed by Western blot analysis (Fig. 1a). COX-2 protein was abundantly expressed in LMeC cells but not expressed in CMeC-1 cells. After treatment with 20 or 50 μM celecoxib for 48 h, expression of COX-2 protein was decreased in LMeC cells (Fig. 1a).

Expression of prostaglandin E2 (PGE$_2$) protein from the supernatant of cell lines was assessed by enzyme-linked immunosorbent assay (Fig. 1b). In the presence of 20 or 50 μM celecoxib, LMeC cells showed a significant decrease of PGE$_2$ protein expression compared with those of control. The values of PGE$_2$ protein level were as follows: untreated (29.2 ± 4.6 pg/ml), 20 μM celecoxib treated (4.63 ± 3.11 pg/ml; $P < 0.001$) and 50 μM celecoxib treated (3.61 ± 3.23 pg/ml; $P < 0.001$). Additionally, the level of PGE$_2$ protein was much lower in CMeC-1 cells than LMeC cells (untreated: 0.34 ± 0.33 pg/ml). With 20 and 50 μM of celecoxib, PGE$_2$ production in CMeC-1 cells did not significantly change ($P = 0.582$ treated with 20 μM of celecoxib, $P = 0.998$ at 50 μM; Fig. 1b). To investigate whether celecoxib affects the proliferation of CMeC-1 and LMeC cells, each cell line was incubated for 48 h with celecoxib. Cell viability and cell surviving fraction were analyzed (Fig. 1c and supplementary Fig. S1 in the online version at doi:10.1016/j.rvsc.2014.03.003). Treatment with celecoxib significantly reduced LMeC cell viability in a dose-dependent manner (Fig. 1c; $P = 0.002$ treated with 20 μM of celecoxib, $P < 0.001$ at 50 μM). In CMeC-1 cells that

lack of COX-2 expression, no significant change of cell proliferation was observed at the presence of 20 μM celecoxib ($P = 0.458$) while cell proliferation was markedly reduced at the 50 μM concentration of celecoxib (Fig. 1c; $P < 0.001$ at 50 μM). Similar results were observed using a clonogenic assay (Supplementary Fig. S1 in the online version at doi: 10.1016/j.rvsc.2014.03.003).

To assess celecoxib-induced anti-proliferative ability, cell cycle analysis was performed (Fig. 2a). The results indicated that celecoxib arrested the cell cycle at the G_0/G_1 phase after 48 h compared to the untreated control. In the CMeC-1, 50 μM celecoxib caused an increase in cells in the G_0/G_1 phase accompanied by a decrease in the G-2 phase (Fig. 2a). In LMeC cells, there was a numerical increase in cells in the G_0/G_1 phase induced by 50 μM of celecoxib although this difference did not show statistical significance (Fig. 2a). To elucidate whether celecoxib may influence cyclin D1 expression associated with a G0–G1 arrest, expression of cyclin D1 was evaluated (Fig. 2b). Celecoxib treatment decreased the levels of cyclin D1 in both cell lines in a dose-dependent manner. In the presence of 50 μM celecoxib, cyclin D1 expression was remarkably lower in CMeC-1 cells than LMeC cells (Fig. 2b).

To determine whether the reduced viability of celecoxib-treated CMeC-1 and LMeC cells was mediated by apoptosis, we analyzed the sub-G_1 phase cell cycle, active caspase-3 expression and inter-nucleosomal DNA fragmentation (Fig. 2c, d). An increased proportion of cells in the sub-G_1 phase of CMeC-1 and LMeC cells were observed (Fig. 2a). The percentage of LMeC cells in the sub-G_1 phase after treatment with 50 μM of celecoxib ($15.26 \pm 4.75\%$) increased compared to the control cells (Fig. 2a, $P = 0.049$). In CMeC-1 cells, the percentage of sub-G1 phase was increased from $5.65 \pm 0.66\%$ to $10.79 \pm 0.62\%$, after treatment of 50 μM celecoxib ($P = 0.021$).

Consistent with the inhibition of cell growth, LMeC cells underwent apoptosis after treatment with 50 μM of celecoxib. The results of Western blot demonstrated that celecoxib treatment induced caspase-3 activation in both LMeC and CMeC-1 cells (Fig. 2c). Interestingly, activation of caspase-3 was markedly higher in LMeC cells more than CMeC-1 cells in the presence of 50 μM celecoxib. DNA fragmentation assay showed apoptotic changes in both melanoma cell lines (Fig. 2d).

One study showed that more than 50% of canine malignant melanomas expressed COX-2 and all oral malignant melanomas expressed the COX-2 protein, on the other hand, only 11 out 20 cutaneous malignant melanomas expressed this enzyme (Pires et al., 2010). The origin of CMeC-1 cell line was canine skin and LMeC cells were derived from canine oral mucosa used in this study (Inoue et al., 2004).

In this study, low dose of celecoxib showed antitumor effects against highly expressing COX-2 cells whereas only high dose of celecoxib showed anticancer effects in COX-2 null cells. These findings are in agreement with a previous report that found different effects of low and high concentration of celecoxib on 3 types of transfected Caco-2 cells, COX-2 overexpressed, COX-2 null and control (to express only very small amounts of COX-2). There were significant differences in sensitivity of celecoxib between them (Maier et al., 2004).

The cell proliferation inhibited as a dose dependent manner in LMeC cells (Fig. 1). However, Western blot analysis showed COX-2 protein expression was lowest after treatment with 20 μM of celecoxib in treated LMeC cells when compared with cells treated with 50 μM (Fig. 1a). A possible explanation for this unexpected finding might be related to a negative feedback loop between COX and COX-produced products. The decrease in COX activity by the COX-2 inhibitor may trigger the production of COX-2 (Lanza-Jacoby et al., 2004). Removal of this negative feedback by celecoxib treatment would result in COX-2 induction. There are similar reports on celecoxib treatment leading to upregulation of COX-2 protein expression in breast cancer cells (Basu et al., 2005).

PGE_2, a product of the enzyme activity of COX on arachidonic acid, has received significant attention for its potential role in carcinogenesis, cancer progression, and metastasis (Herschman, 1996; Mohammed et al., 2001a). High concentrations of PGE_2 were found in some types of naturally occurring cancers, and approximately half of the investigated oral melanoma samples showed elevated PGE_2 production (Fig. 1b; the mean PGE_2 concentration in canine melanoma: 209 ng/g) (Mohammed et al., 2001a). In the current study, PGE_2 was high in LMeC cells along with COX-2 level. Both doses of celecoxib significantly reduced PGE_2 secretion of LMeC cells (Fig. 1b), indicating that celecoxib is a potent inhibitor of COX-2-induced PGE_2 production. These findings suggest that COX-2 plays a crucial role in the production of PGE_2 and that celecoxib successfully suppresses the proliferation in canine melanoma cells that express high levels of COX-2.

With a high concentration of celecoxib treatment, the highly COX-2 expressing cell line mainly underwent apoptosis, while the COX-2 null cell line mostly underwent cell cycle arrest (Fig. 2). These findings agree with the results of a previous study. In previous study, celecoxib induced apoptosis in MDA-MB-231 cells that express high levels of COX-2, whereas celecoxib induced cell cycle arrest in MDA-MB-468 cells that express low levels of COX-2 (Basu et al., 2005). In other study, COX-2 overexpressed Caco-2 cells and control Caco-2 cells were more sensitive to the apoptosis induction potency of celecoxib than COX-2 non-expressed Caco-2 cells (Maier et al., 2004). However, another study showed that celecoxib induces apoptosis in human melanoma cell lines regardless of COX-2 protein expression level (Bundscherer et al., 2008). In the present study, the antitumor effect of high dose of celecoxib seemed to be independent of COX-2 expression of the cancer cell. Although there were different degrees, an apoptotic effect was observed in both CMeC-1 and LMeC cell lines treated with high concentration of celecoxib (50 μM). Similar effects of celecoxib have been observed in rat prostate cancer cell lines (Narayanan et al., 2003; Patel, 2005).

However, other investigations showed celecoxib had no significant influence on COX-2-deficient and low expressed cancer cell lines (El-Rayes et al., 2004; Wu et al., 2003). If the anticancer effects of celecoxib against COX-2 negative tumor were in doubt, tumor detection and characterization of COX-2 expression might be useful for COX-2 targeted treatment. Alternatively, PGE_2 concentration of naturally occurring canine cancer also could be evaluated (Mohammed et al., 2001b).

In a recent study, celecoxib stimulated tumor cell invasion and chemoresistance in non-small cell lung cancer (NSCLC) regardless of COX-2 status (Wang et al., 2012). However, according to a phase II study by Gogas, the combination of temozolomide and celecoxib is safe and potentially effective in the treatment of metastatic melanoma in people (Gogas et al., 2006). In this phase II study, expression of COX-2 was noted in melanoma cells in all examined cases (Gogas et al., 2006). Furthermore, feeding celecoxib to transgenic adenocarcinoma of the mouse prostate model (TRAMP) resulted in suppression of prostate carcinogenesis and complete absence of metastasis, along with increased long-term survival. In TRAMP mice, levels of COX-2 enzyme activity and protein expression were significantly higher than in nontransgenic mice (Gupta et al., 2004). In our study, high dose of celecoxib showed anticancer effect in both melanoma cell lines. Furthermore, COX-2 positive LMeC cells were more sensitive to celecoxib treatment than COX-2 null cells, CMeC cells. Therefore, the anticancer effect of celecoxib remains controversial. However, different tumors are not equally sensitive or responsive to chemotherapeutic drugs. A previous study by Lotan described that each cancer cell lines from melanoma and breast carcinoma may respond differently to retinoic acid therapy (Lotan, 1979). According to a study by Lei, 3 subtypes of gastric adenocarcinoma were identified. Moreover the subgroups have differences in molecular, genetic features and response to therapy (Lei et al., 2013). Different types of cancer have different target molecules for cancer therapy and different susceptibilities to anticancer agents. Accordingly, it will be necessary to further investigate the mechanism of anticancer effect whether it is related to COX-2 and PGE_2. Otherwise, further study is essential for finding a signal pathway interfering with the anticancer effect of celecoxib in certain types of cancer. Or further study is essential for finding a signal pathway interfering with the anticancer effect of celecoxib in certain type of cancer.

In conclusion, we have found that celecoxib treatment suppressed the growth of both canine melanoma cell lines, and mainly induced apoptosis with high dose of celecoxib in COX-2 positive LMeC cells and cell cycle arrest in COX-2 null CMeC-1 cells. Moreover, LMeC cells showed more sensitivity to celecoxib than CMeC-1 cells. These results indicate the possible clinical importance of celecoxib as a good candidate agent for treating canine malignant melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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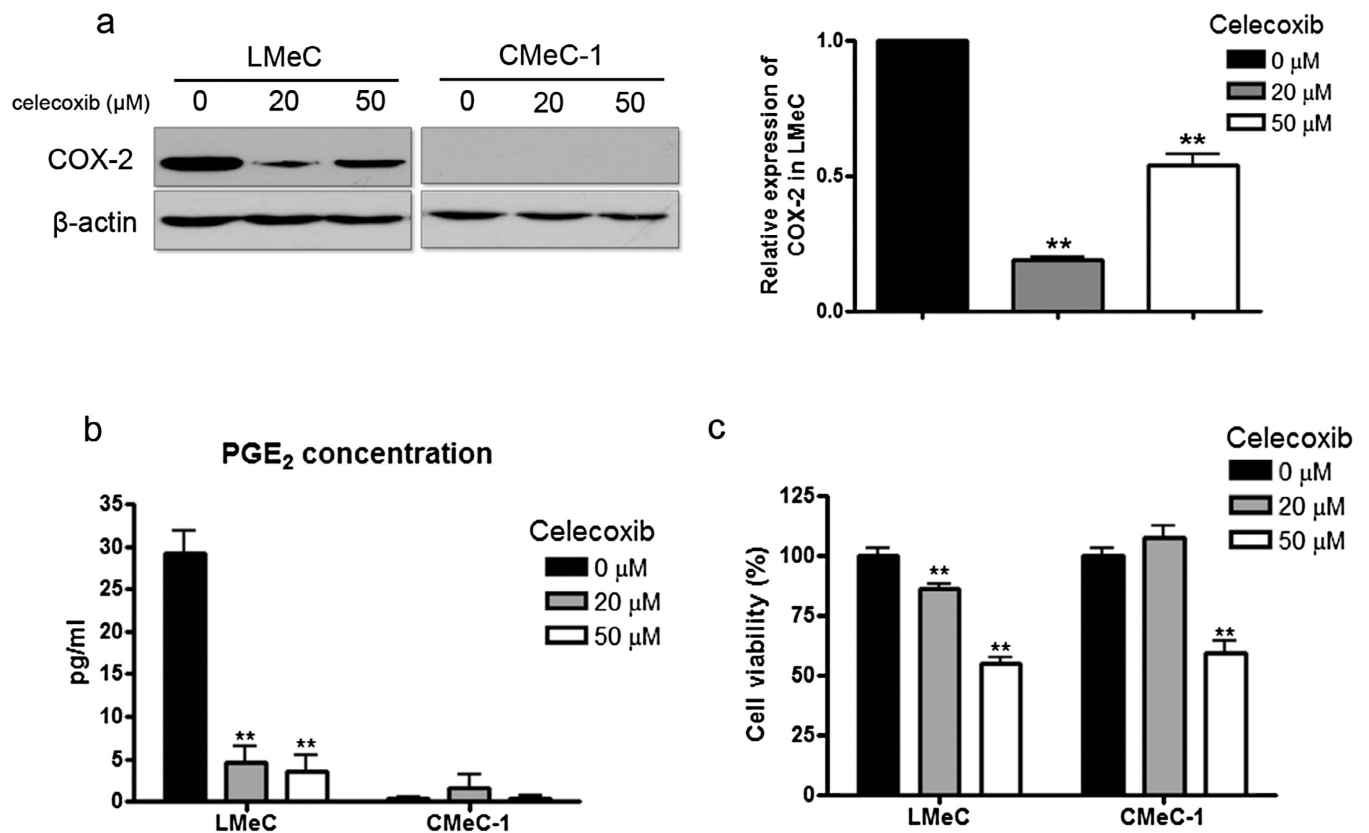
Fig. 1.

The effects of celecoxib on COX-2 expression in canine maligmant melanoma and the inhibitory effects of celecoxib on cell proliferation. (a) Western blot analysis of COX-2 expression in LMeC and CMeC-1 canine melanoma cell lines. COX-2 expression was observed in LMeC cells and was absent in CMeC-1 cells. Celecoxib reduced COX-2 expression in LMeC cells. (b) Effects of celecoxib on production of COX-2's principal metabolic product, PGE_2. Cells were treated with celecoxib (20 and 50 μM) for 48 h. CMeC-1 cells did not produce any PGE_2. (c) The growth rates of LMeC and CMeC-1 cells measured by an MTT assay after treatment celecoxib. Proliferation was inhibited when LMeC were treated with 20, and 50 μM celecoxib. In CMeC-1 cells, the 50 μM celecoxib inhibited cell proliferation. Data are presented as the mean ± SD; **$P < 0.01$.
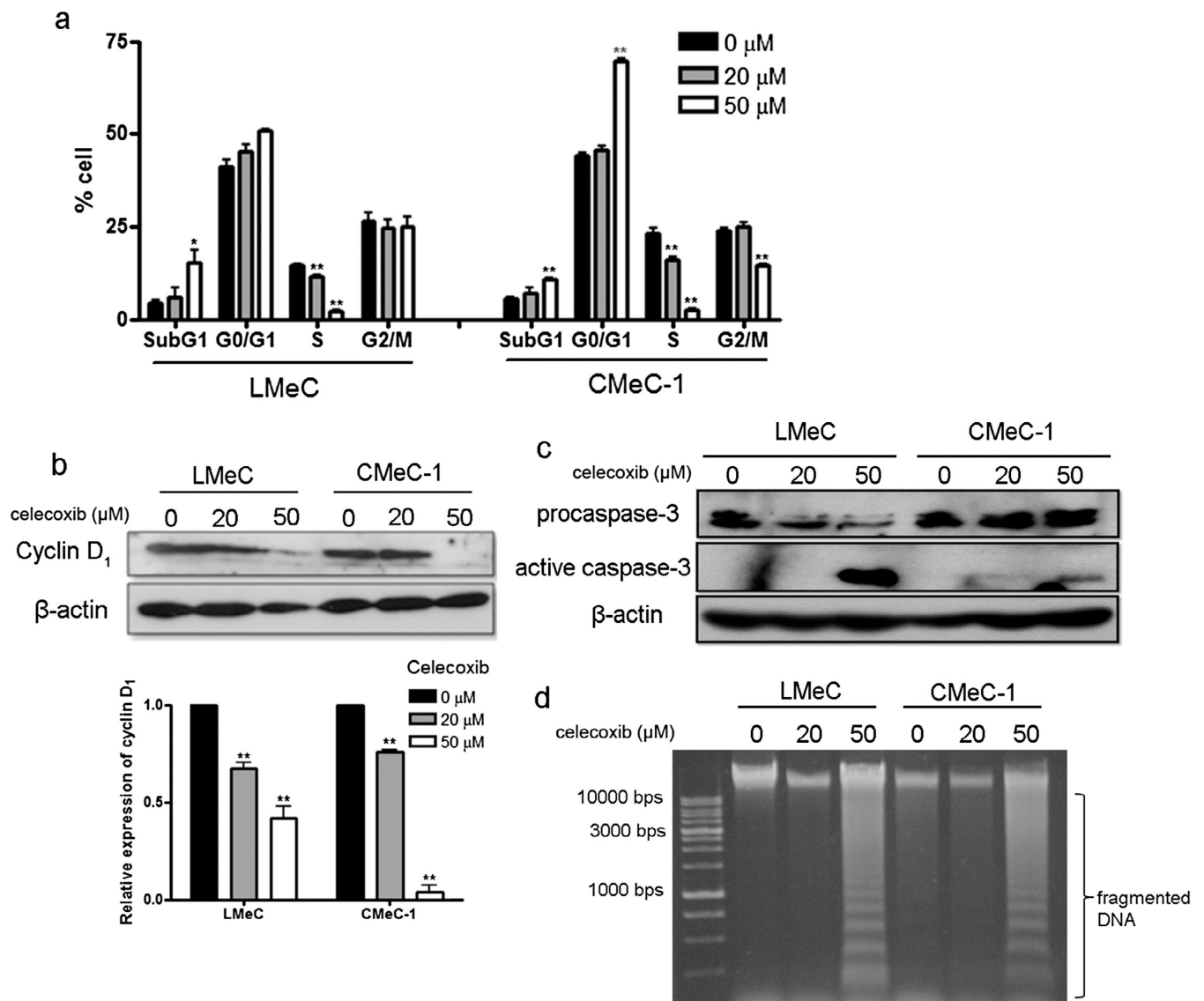
Fig. 2.
Celecoxib induced G1-S arrest and apoptosis in canine malignant melanoma cell lines. (a) Representative flow cytometry results from LMeC and CMeC-1 cell after 48 h of incubating with various concentration of celecoxib (0, 20, and 50 μM). Increases in the proportions of cells in the G0/G1 phase of the cell cycle and decreases in the S phase were observed (*$P <$ 0.05, **$P <$ 0.01). (b) Cells were treated with celecoxib subjected to Western blot with specific antibodies directed against cyclin D1. The 50 μM celecoxib reduced cyclin D1 expression of CMeC-1 cells. (c) The results of caspase-3 activation. Celecoxib treatment induced caspase-3 activation in both LMeC and CMeC-1 cells. Activated form of caspase-3 was higher in LMeC cells more than CMeC-1 cells when 50 μM celecoxib was treated. (d) DNA fragmentation (*$P <$ 0.05, **$P <$ 0.01). The typical DNA ladder patterns were showed in LMeC cells and CMeC-1 cells when they were incubated with 50 μM celecoxib.