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COX-2 inhibits anoikis by activation of the PI-3K/Akt pathway in human bladder cancer cells

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Abbreviations: COX-2, cyclooxygenase-2; NGF, nerve growth factor; PGE_2, prostaglandin E_2

Abstract

Cyclooxygenase-2 (COX-2) has been reported to be associated with tumor development and progression as well as to protect cells from apoptosis induced by various cellular stresses. Through a tetracycline-regulated COX-2 overexpression system, we found that COX-2 inhibits detachment-induced apoptosis (anoikis) in a human bladder cancer cell line, EJ. We also found that the inhibition of anoikis by COX-2 results from activation of the PI-3K/Akt pathway as evidenced by suppression of the COX-2 effect on anoikis by a PI-3K inhibitor, LY294002. Furthermore, COX-2 enhanced Mcl-1 expression in the anoikis process, implying that Mcl-1 also may be involved in mediating the survival function of COX-2. Together, these results suggest that COX-2 inhibits anoikis by activation of the PI-3K/Akt pathway and probably by enhancement of Mcl-1 expression in human bladder cancer cells. This anti-anoikis effect of COX-2 may be a part of mechanisms to promote tumor development and progression.

Keywords: anoikis; bladder; cancer; COX-2; Mcl-1; PI-3K/Akt

Introduction

Prostaglandin endoperoxide H synthases (PGHSs) catalyze the conversion of arachidonic acid and O_2 to PGH2, the committed step in prostanoid biosynthesis. Two isoforms of PGHS, PGHS-1 (COX-1) and PGHS-2 (COX-2), have been identified. COX-1 is responsible for basal and constitutive prostaglandin synthesis, whereas COX-2 is an inducible enzyme of which expression can be rapidly increased in response to various stimuli (Smith *et al.,* 1996).

COX-2 has been known to act as a survival factor under a variety of cellular stress conditions. COX-2 protects human normal cells such as neurons, cardiomyocytes, renal cells, mammary epithelial cells, fibroblasts and endothelial cells from apoptosis induced by various stresses including NGF-withdrawal, ischemia, hypertonicity or DNA damaging agents, of which the mechanisms are not known yet (Chang *et al.,* 2000; Dowd *et al.,* 2000; Yang *et al.,* 2000; Han *et al.,* 2002). In addition to these normal primary cells, COX-2 also has been known to protect cancer cells from apoptosis through regulation of Bcl-2 family protein expression. In human colon cancer and cholangiocarcinoma cells, COX-2 inhibited apoptosis by up-regulation of Bcl-2 and Mcl-1 expression, respectively (Nzeako *et al.,* 2002; Tang *et al.,* 2002). In human lung cancer cells, COX-2 repressed apoptosis by activation of the PI-3K/Akt → Mcl-1 pathway (Lin *et al.,* 2001).

It is well documented that COX-2 expression correlates with tumor development and progression in most of human tumors. The levels of COX-2 expression are not only higher in cancer tissues than in normal tissues, but also strongly correlate with local invasion and metastasis (Kundu *et al.,* 2001; Ohno *et al.,* 2001; Yoshimura *et al.,* 2001). Moreover, COX-2 selective inhibitors have been reported to inhibit tumor development in colons, breasts or urinary bladders (Grubbs *et al.,* 2000; Steinbach *et al.,* 2000; Orengo *et al.,* 2002). Nevertheless, the exact action mechanisms of COX-2 in tumor development or progression remain to be elucidated.

Anoikis is a form of apoptosis triggered when cells lose their adhesion to the extracellular matrix. Anoikis functions as a surveillance mechanism to preserve normal tissue architecture by eliminating cells attempting to deviate from their normal spatial constraints

(Frisch *et al.,* 1997). It means that as cells become transformed and have the capacity for invasion and metastasis, they must acquire the resistance to anoikis.

In the present study, we demonstrated that COX-2 inhibits anoikis in EJ human bladder cancer cells, which is reversed by the treatment of NS-398 or LY294002. COX-2 induced Akt phosphorylation as well as Mcl-1 expression in the anoikis condition. These results suggest that COX-2 can act as a survival factor when cells lose their adhesion to the extracellular matrix by controlling the major anti- apoptotic PI-3K/Akt pathway and the Mcl-1 protein expression.

Materials and Methods

Generation of EJ-COX-2 and cell culture

pTet-COX-2 was constructed by subcloning COX-2 cDNA downstream of the tetracycline-regulated promoter into pDS1293 which contains *neo*^{r}. EJ-ETH cells (Sugrue *et al.,* 1997) containing tTA expression cassette were transfected with pTet-COX-2 using Lipofectamine2000 reagent (Life technologies Inc., Grand Island, NY). Transfectants were selected in the presence of tetracycline (1 μg/ml), hygromycin (100 μg/ml) and geneticin (400 μg/ml). Individual clones of stable transfectants, named EJ-COX-2, were maintained in the presence of tetracycline (1 μg/ml) in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml), hygromycin (100 μg/ml) and geneticin (400 μg/ml). To induce COX-2 expression, EJ-COX-2 cells were first rinsed three times with PBS and then switched to the culture media lacking tetracycline.

Anoikis induction and trypan blue exclusion assay

To induce anoikis, cells were plated on tissue culture dishes coated with 10 mg/ml poly-HEMA (Sigma Aldrich, St. Louis, MO) or 1% agarose (Life technologies Inc., Grand Island, NY). Poly-HEMA was dissolved in ethanol as described previously (Zhu *et al.,* 2001). After the anoikis induction, cells were collected and mixed with trypan blue solution and then both of live and dead cells were counted.

Western blot analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris-HCl, 1% Tween-20, 1% sodium deoxycholate and 0.1% SDS) with 0.5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 μg/ml pepstatin. Proteins were resolved in SDS-PAGE and transferred to nitrocellulose membranes, which were probed with the appropriate antibodies. The immunoreactive protein complexes were detected by enhanced chemiluminescence (Amersham Bioscience, Boston, MA). The specific antibodies for p-Akt (Ser473), Akt, Mcl-1, Bcl-2, Bcl-xL, Bax, Bak and cleaved caspase-3 were obtained from Cell Signaling technology. Anti-Cox-2 antibody was purchased from BD Biosciences (San Jose, CA).

PGE_2 assay

The amount of PGE_2 secreted in the culture medium was determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). The production of PGE_2 was normalized to protein concentrations.

Results

COX-2 overexpression inhibits anoikis in human bladder cancer EJ cells

In order to investigate the effect of COX-2 on anoikis, we generated a stable cell line of EJ, EJ-COX-2, expressing COX-2 under the control of a tetracycline (tet)-regulated promoter. The expression of COX-2 was increased by tetracycline removal in EJ-COX-2 cells (Figure 1A). In addition, the level of prostaglandin E_2 (PGE_2), one of the major enzymatic products of COX-2, was also increased significantly after tetracycline removal in EJ-COX-2 cells (Figure 1B). To test whether COX-2 expression affects the cellular

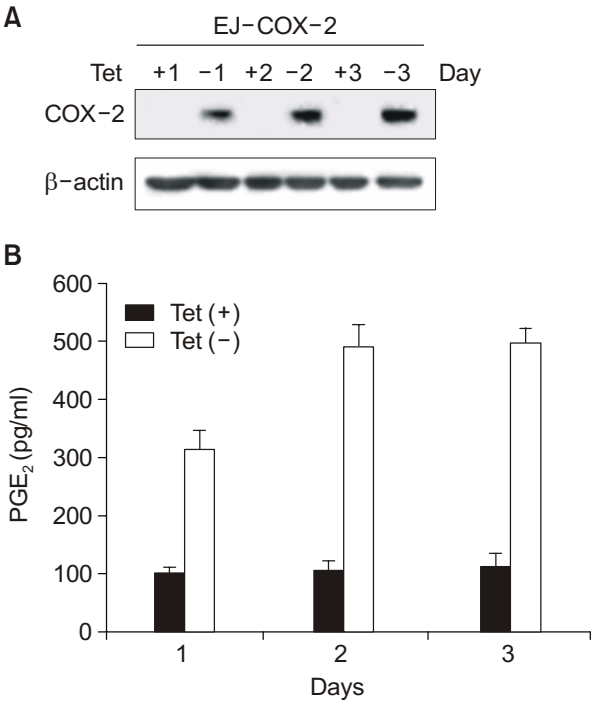

Figure 1. Induction of COX-2 expression and PGE_2 production in EJ-COX-2 cells. (A) COX-2 protein was induced by tetracycline removal in EJ-COX-2 cells. β-actin was used as a loading control. (B) Prostaglandin E_2 (PGE_2) level was increased in response to COX-2 induction in EJ-COX-2 cells. Cells were grown in the presence or absence of tetracycline for 1, 2, or 3 day. Culture media were collected and analyzed for PGE_2 production using enzyme-linked immunosorbent assay.

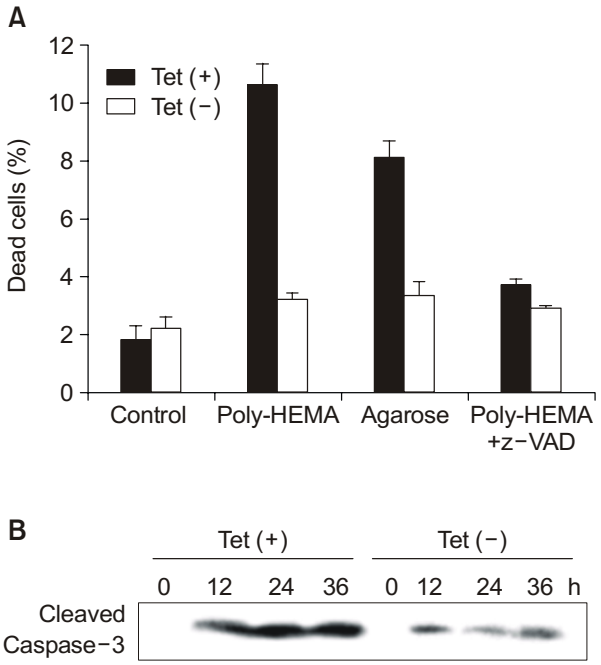

Figure 2. Inhibition of anoikis by COX-2 overexpression. EJ-COX-2 cells were cultured in the presence or absence of tetracycline for two days and plated on regular, poly-HEMA-coated or 1% agarose-coated plates. Z-VAD-fmk (50 μM) was pretreated for 1 h. (A) Cells were harvested after 24 h for trypan blue staining. The percentage of dead cells were calculated and compared. Error bars indicate means ± SD of three independent experiments with duplicate plates. (B) Total cell extracts were isolated for Western blot analysis at the in dicated time points. Note that the cleaved form of caspase-3 is diminished by COX-2 expression.

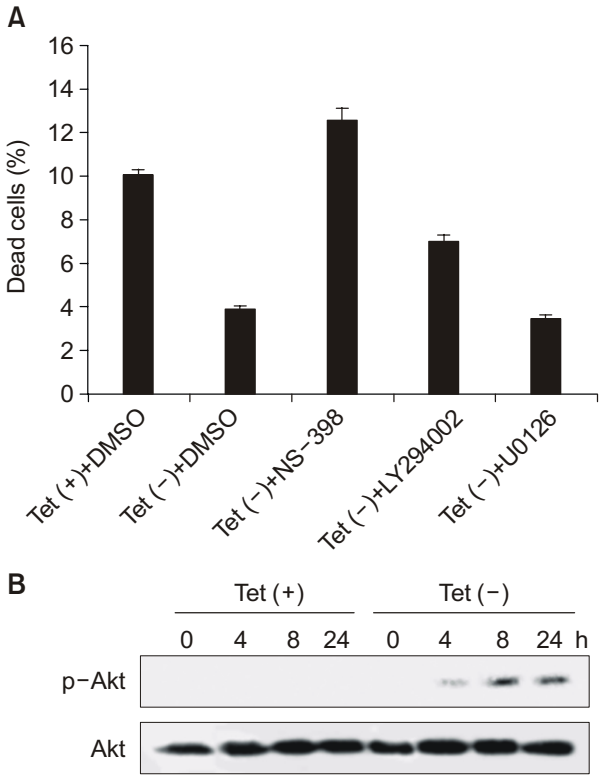

Figure 3. The PI-3K/Akt pathway mediates anti-anoikis effect of COX-2. EJ-COX-2 cells were cultured in the presence or absence of tetracycline for two days and plated on poly-HEMA-coated plates. (A) The same volume of DMSO (vehicle), NS-398 (20 uM), LY294002 (50 uM) or U0126 (50 uM) was added when cells were plated. The trypan blue assay was done after 24 hours and the percentage of dead cells were calculated. Error bars indicate means ± SD of three independent experiments with duplicate plates. (B) Total cell extracts were isolated at the indicated time points and western blot analysis was performed using antibodies against p-Akt (Ser473) and Akt.

sensitivity to anoikis, EJ-COX-2 cells were cultured in tissue culture dishes coated with poly-HAMA or 1% agarose to prevent cell attachment to the substratum. As shown in Figure 2A, COX-2 overexpression reduced cell death rates from ~11% to ~3% in poly-HEMA-coated dishes and from ~8% to ~3% in agarose-coated dishes. Moreover, a selective COX-2 inhibitor, NS-398, suppressed the anti-anoikis effect of COX-2 completely, demonstrating that the COX-2 effect was mediated by the enzymatic function of COX-2 (Figure 3). Addition of the general caspase inhibitor, z-VAD-fmk, blocked the detachment-induced cell death almost completely (Figure 2A) and the detachment-induced caspase-3 cleavage was diminished by COX-2 overexpression (Figure 2B), confirming that the cell death caused by suspension cultures was due to a caspase-dependent apoptosis pathway. Taken together, all these data demonstrate that COX-2 overexpression inhibits anoikis of EJ cells.

COX-2 inhibition of anoikis is mediated by activation of the PI-3K/Akt pathway

To investigate the mechanism by which COX-2 inhibits anoikis, we first checked PI-3K/Akt and Ras/Raf/ERK pathways, which have been known as major cellular survival signaling pathways. As shown in Figure 3A, the PI-3K inhibitor LY294002 inhibited the anti-anoikis effect of COX-2 by 50%, while the MEK1/2 inhibitor U0126 had no significant effect. In addition, COX-2 increased Akt phosphorylation in the anoikis process (Figure 3B). These results indicate that the PI-3K/Akt pathway is activated by COX-2 in the anoikis process, which plays a major role in mediating the anti-anoikis function of COX-2.

Mcl-1 expression is enhanced by COX-2 overexpression in the anoikis process

To elucidate further the mechanism by which COX-2 inhibits anoikis, we next examined the expression levels of Bcl-2 family proteins that have been reported to be ultimate effectors mediating the anti-apoptotic functions of COX-2 as well as PI-3K/Akt. As shown in Figure 4, Mcl-1 expression was increased about 4-fold by COX-2 overexpression in the anoikis pro-

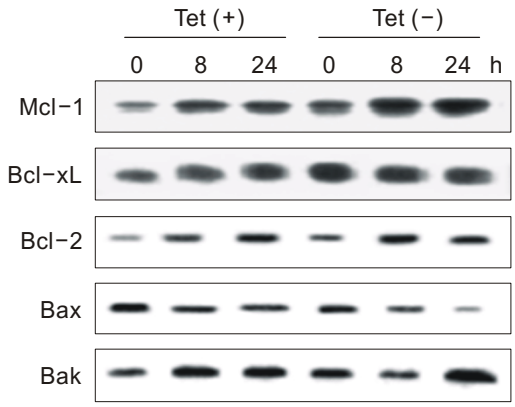

Figure 4. Enhanced Mcl-1 expression by COX-2 in the anoikis process. EJ-COX-2 cells were cultured in the presence or absence of tetracycline for two days and plated on poly-HEMA-coated plates. Total cell extracts were isolated at the indicated time points and Western blot analysis was carried out using antibodies against Bcl-2 family proteins including Mcl-1, Bcl-xL, Bcl-2, Bax and Bak.

cess. In contrast, no significant changes were observed in the expression levels of other Bcl-2 family proteins such as Bcl-xL, Bcl-2, Bax and Bak (Figure 4). These findings suggest that Mcl-1 may also be involved in mediating the anti-anoikis function of COX-2.

Discussion

Apoptosis is triggered when cells lose their adhesion to the extracellular matrix. This anoikis phenomenon is critical to maintain the tissue architecture and to prevent tumor development and progression. Though the mechanisms of anoikis are not completely understood yet, recent reports suggest that deprivation of integrin-mediated survival signals is one of the major causes of anoikis. Cellular adhesion to the matrix is mainly mediated by integrins, which provide survival signals through activation of the PI-3K/Akt, Ras/Raf/Erk or JNK pathways. Among them Akt seems to be a central molecule of the cellular survival signaling because FAK and ILK, both of which are integrin-associated non-receptor kinases, converge to the activation of Akt. Akt executes its survival function, in part, by inhibition of caspase-9 and Bad (Grossmann, 2002).

Previously, there was a report that hepatocyte growth factor inhibits anoikis by induction of COX-2 in human head and neck cancer UMSCC1 cells, of which the mechanism remained to be explained (Zeng *et al.,* 2002). In the present study we demonstrated that COX-2 inhibits anoikis by activation of the PI-3K/Akt pathway in human bladder cancer EJ cells, which provides one of the mechanisms for the anti-anoikis effect of COX-2 (Figure 2 and 3). Nevertheless, the fact that a PI-3K inhibitor could not reverse the anti-anoikis effect of COX-2 fully suggests that further studies are required to understand the survival function of COX-2 completely (Figure 3A).

COX-2 has been known to inhibit apoptosis in human cancer cells through regulation of Bcl-2 family protein expression (Lin *et al.,* 2001; Nzeako *et al.,* 2002; Tang *et al.,* 2002). Especially in human lung cancer cells COX-2 inhibited apoptosis through the PI-3K/Akt → Mcl-1 pathway (Lin *et al.,* 2001). In the present study we also found that COX-2 increases Mcl-1 expression (Figure 4), suggesting that COX-2 may inhibit anoikis by activation of the PI-3K/Akt → Mcl-1 pathway.

Accumulating evidence indicates that COX-2 plays an important role in tumor development and progression. In several epidemiological studies, regular administration of COX-2 inhibitors reduced risk of colon cancer development (Gridley *et al.,* 1993; Steinbach *et al.,* 2000; Phillips *et al.,* 2002). The present study demonstrating that COX-2 inhibits anoikis by PI-3K/Akt activation provides the molecular basis of the anti-tumorigenesis effect of COX-2 inhibitors.

Acknowledgment

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