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Interrelation of COX-2 inhibition on expression of vascular endothelial growth factor and interleukin-8 in lung cancer cells

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Abstract

Cyclooxygenase 2(COX 2) Selective COX-2 inhibitors are a type of non-steroidal anti-inflammatory drug (NSAID) that directly targets cyclooxygenase-2, COX-2, an enzyme responsible for inflammation and pain. Targeting selectivity for COX-2 reduces the risk of peptic ulceration, and is the main feature of celecoxib, rofecoxib and other members of this drug class. After several COX-2 inhibiting drugs were approved for marketing, data from clinical trials revealed that COX-2 inhibitors caused a significant increase in heart attacks and strokes, with some drugs in the class having worse risks than others. Rofecoxib (commonly known as Vioxx) was taken off the market in 2004 because of these concerns and celecoxib and traditional NSAIDs received boxed warnings on their labels.

Keywords: cyclooxygenase, Interleukin, Western blotting, small cell lung cancer (SCLC), In silico analysis

Introduction

Methods

The levels of IL-8, VEGF and prostaglandin E₂ (PGE₂) were measured by ELISA. Expression of COX-1 and COX-2 was determined by Western blotting. Inhibition or knockdown of COX-2 was achieved by treating NSCLC cells with specific COX-2 inhibitor NS-398 or COX-2 siRNA, respectively.

Results

We found that NSCLC cell lines produced more IL-8 than VEGF ($p < 0.001$). In contrast, small cell lung cancer (SCLC) cell lines produced more VEGF than IL-8 ($p < 0.001$). COX-1 was expressed in all cell lines, but COX-2 was expressed only in NSCLC cell lines. Consistent with this, PGE₂ was significantly higher in NSCLC cell lines than SCLC cell lines ($p < 0.001$). We tested these cell lines with a potent specific COX-2 inhibitor NS-398 at concentrations of 0.02, 0.2, 2, 20 μ M for 24 or 48 h. The COX-2 activity was reduced in a dose-dependent fashion as shown by reduced PGE₂ production. VEGF was significantly reduced following the treatment of NS-398 in A549 (by 31%) and MOR/P (by 47%) cells lines which expressing strong COX-2, but not in H460 cell line which expressing very low COX-2. However, IL-8 was not reduced in these cell lines. To confirm these results, we knocked down COX-2 expression with COX-2 siRNA in these cell lines. VEGF was significantly decreased in A549 (by 24%) and in MOR/P (by 53%), but not in H460 whereas IL-8 was not affected in any cell line.

Conclusion

We conclude that NSCLC cells produce much higher levels of IL-8 than SCLC cells whereas both NSCLC and SCLC cells produce similar levels of VEGF. COX-2 is only expressed in NSCLC cells, but not in SCLC cells. VEGF is produced in both NSCLC and SCLC cells regardless of COX-2 expression. However, VEGF production is, at least partly, COX-2 dependent in NSCLC cells expressing COX-2. In contrast, IL-8 production is COX-2 independent in both NSCLC and SCLC cells. We speculate that combined targeting of COX-2 and IL-8 may be useful in the treatment of patients with NSCLC and targeting VEGF may be useful in the treatment of patients with SCLC.

Background

Lung cancer remains the leading cause of cancer death in many countries worldwide. There is considerable interest in anti-angiogenic drugs as therapeutic agents for lung cancer.

Angiogenesis in tumours is promoted through the secretion of a variety of pro-angiogenic factors. Among these, vascular endothelial growth factor (VEGF) is important in many tumour types due to both its potent activity and markedly elevated expression level. Several studies have shown that high levels of VEGF are associated with increased tumour vascularity, advanced stage and poor prognosis in patients with non-small cell (NSCLC) and small cell lung cancer (SCLC) [1, 2, 3, 4, 5]. In addition to its angiogenic effects, functional VEGF receptors are expressed on SCLC cells and VEGF induces cell proliferation and migration in these cells [6]. Treatment with a humanized monoclonal antibody to VEGF, bevacizumab (Avastin), prolongs the survival of patients with NSCLC [7].

1. Interleukin-8 (IL-8), one of the ELR⁺ CXC family of chemokines, is another potent pro-angiogenic factor and its expression is associated with angiogenesis, tumour progression and survival in patients with NSCLC [8, 9, 10, 11]. In addition to its angiogenic effects, IL-8 receptors (CXCR1 and CXCR2) are expressed on lung cancer cells and IL-8 can act as growth/survival factor to these cells [12]. Hence, both VEGF and IL-8 contribute to lung cancer progression through angiogenic and direct mitogenic effects [13, 14]. The relative contributions of different angiogenic factors to lung cancer growth are unknown.

Cyclooxygenases (COX) are key enzymes in the conversion of arachidonic acid to prostaglandin (PG) and other eicosanoids including PGE₂, PGD₂, PGF_{2α}, PGI₂ and thromboxane A₂. COX-1 is present in nearly all cells whereas COX-2 is normally undetectable but is inducible under circumstances such as inflammation and cancer. Cancer cells, including NSCLC cells, express high levels of COX-2 protein [15, 16, 17]. COX-2 overexpression has been associated with poor prognosis in NSCLC, although a recent meta-analysis challenges this [18]. It is correlated to VEGF and IL-8 expression in NSCLC [19, 20]. Selective COX-2 inhibitors have been shown to inhibit the growth and metastasis of several types of cancers [21]. Celecoxib (Celebrex) and rofecoxib (Vioxx) have been tested in clinical trials, but their utilities are limited by cardiac adverse effects. Nevertheless, COX-2 could be a potential target to limit lung cancer growth. However, the mechanism underlying inhibition of angiogenesis and metastasis by targeting COX-2 is not fully understood. The aim of this study was to establish whether there is a direct relationship between COX-2 expression and VEGF and IL-8 production in lung cancer cells.

Methods

Lung cancer cell lines, cell culture and reagents

The lung cancer cell lines used were A549, H460, MOR/P (NSCLC) and GLC19, H69, H345, H711, Lu165 (SCLC). All cell lines were grown in RPMI 1640 (Bio Whittaker) with 10% fetal calf serum (Biosera) at 37°C in 5% CO₂, 95% air. To collect supernatants for VEGF and IL-8 detection, NSCLC (adherent) cells were seeded in 6 well tissue culture plates and SCLC (suspension) cells were seeded in 24 well plates before being treated with various concentrations of the COX-2 inhibitor NS-398 (Cayman Chemical and Calbiochem) in triplicate for 24 h or 48 h. Anti-COX-1 and anti-COX-2 antibodies were purchased from Cayman Chemical. Anti-actin antibody was purchased from Sigma. Antibodies to VEGF and IL-8 for ELISA were purchased from R&D systems. PGE₂ enzyme immunoassay kit was purchased from R&D systems. The siRNA-COX-2 and siRNA-control were purchased from Dharmacon.

Enzyme-linked immunosorbent assay (ELISA)

Total VEGF and IL-8 concentrations were determined by ELISA kits as previously described for IL-8 [12]. PGE₂ was measured using a highly sensitive PGE₂ competitive ELISA kit according to the manufacturer's instruction. The intensity of colour developed was measured using a Dynatech MR5000 microplate reader at 450 nm optical density (OD) with correction at 570 nm.

Western blotting

Whole cell lysates were prepared by resuspending cell pellets in CellLytic^{TMM} lysis buffer (Sigma) and incubating on ice for 15 minutes before centrifuging at 13,000 rpm for 15 minutes. Protein concentrations were measured in triplicate with the quick start Bradford reagent (Bio-Rad), and 20 µg of protein was added to loading buffer (62.5 mM Tris.HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT and 0.01% bromophenol blue), boiled, and electrophoresed on a 12% polyacrylamide/SDS gel, before being transferred to a Hybond P membrane (Amersham Biosciences). Membranes were incubated in primary anti-COX-2 (1:1000 dilution) or anti-COX-1 antibody (1:200 dilution) for 2 h at room temperature with gentle agitation, and in peroxidase-conjugated rabbit anti-mouse IgG for 2 hour at room temperature before being treated with the ECL detection system and exposed to hyperfilm ECL film (Amersham Biosciences). In some experiments, the membranes were washed and re-probed with anti-actin antibody as control of equal loading.

Cell growth assay

Cell growth was determined by direct cell counting under the microscope after cells were treated with various concentration of NS-398 for 24 or 48 h. The cells were trypsinized and resuspended in PBS. 50 µl of cell suspension was added to 50 µl of 0.4% Trypan Blue solution, mixed thoroughly and left for 10 min. Viable cells and dead cells (staining blue) were counted in four 1 mm corner squares of a hemocytometer slide. Total cells = (average count/square) × (dilution factor) × 10⁴ × (predilution volume).

siRNA transfection

On-target COX-2 plus SMARTpool (COX-2 siRNA) and SiCONTROL Non-Targeting siRNA#1 (control siRNA) were purchased from Dharmacon. The sequences of On-target COX-2 plus SMARTpool (COX-2 siRNA) [GenBank: NM_000963] were GGACUUAUGGGUAAUGUUUU (duplex 6); GAUAAUUGAUGGAGAGAUGUU (duplex 7); GUGAAACUCUGGCUAGACAUU (duplex 8) and CGAAAUGCAAUUAUGAGUUUU (duplex 9). The siRNAs were transfected into the cells in 12-well plate using DharmaFECT1 transfection reagent according to manufacturer's instructions. Four hours after transfection, the media was replaced by fresh media with 10% FCS. After further 48 h, the media were collected for ELISA and cells were collected for protein extraction.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). Statistical significance was obtained using unpaired student's t-test. Each experiment was repeated at least 3 times. Values with p < 0.05 were considered significant.

“In Silico” analysis of COX-2 metabolism as well as Interleukin-8(IL-8) metabolism:

Paying regards to recent updates of analytical systems we have

used “in silico” approach of analysis of human COX-2 as well as IL-8 metabolism. This approach clearly shows the interrelation of COX-2 in the metabolic cycle in human body.

In the following figure, we have shown this consequences and the structure of COX-2 and it clearly interprets COX-2 mediates its activity.

FASTA ▾

cyclooxygenase-2 [Homo sapiens]

GenBank: AAA58433.1

[GenPept](#) [Identical Proteins](#) [Graphics](#)

>gi|181254|gb|AAA58433.1| cyclooxygenase-2 [Homo sapiens]

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MLARALLLCAVLALSHLANPCCSHPCQNRGVCMSVGFQDKDCDTRTGFYGENCSTPEFLTRIKLFLKPT
PNTVHYLLTHFGKFWNVVNNIPFLRNAIMSYVLTSRSHLIDSPPTYNADYGYKSMWAFSMLSYTRALPP
VPDDCPTPLGVKGGKQLPDSNEIVGKLLLRKFLPDPQGSNMMFAFFAQHFTHQFKFDHHRGPAFTNGL
GHGVDLNHIYGETLARQRKRLRFKDGKMKYQIIDGEMYPPTVKDQAEHMIYPPQVPEHLRFVAVGQEVFGL
VPGLMMYATIWLRHNRVCDVLKQEHPEWGDQLFQTSRLILIGETIKIVIEDYVQHLSGYHFKLKFDP
LLFNKQFYQNRIAAEFNTLYHWHPLLPDFTFIHQDKYNYQQFIYNNISILLEHGITQFVESFTRQIAGR
VAGRRNVPVAVQKVSQASIDQSRQMKYQSFNEYRKRFLKPKYESFEELTGEKMSAELEALYGDIDAVELY
PALLVEKRPDAIFGETHVEVGAPFSLKGLMGVNICSPAYWKPSTFGGVEVGFQIINTASIQSLICMNVKG
CPTFSFVDPPELIKVTVINASSRSGLDDINPTVLLKERSTEL
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Fig 1: Human COX-2 Protein Sequence

"COX2" redirects here. For the cyclooxygenase isoenzyme, see COX-2. Cytochrome c oxidase subunit 2, also known as cytochrome c oxidase polypeptide II, is a protein that in humans is encoded by the MT-CO2 gene. Cytochrome c

oxidase subunit II, abbreviated COXII, COX2, COII, or MT-CO2, is the second subunit of cytochrome c oxidase. The following are the metabolic pathways of Human COX-2 [Fig-2 A] and [2 B].

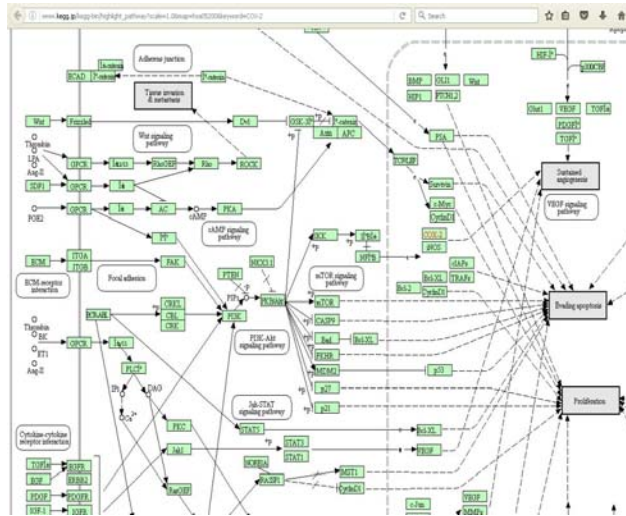


Fig 2: A describes the metabolic pathway of COX-2 in human body (Part-1)

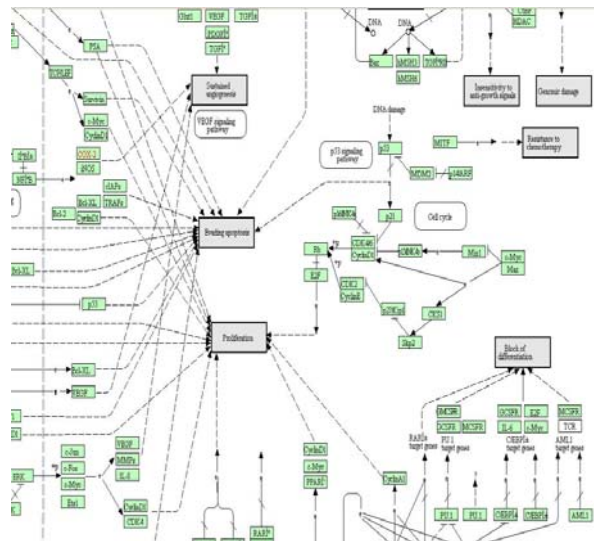


Fig 2: B demonstrates the COX-2 metabolism in human body. (Part 2)

COX-2 Pathway-- Human Lung Cancer

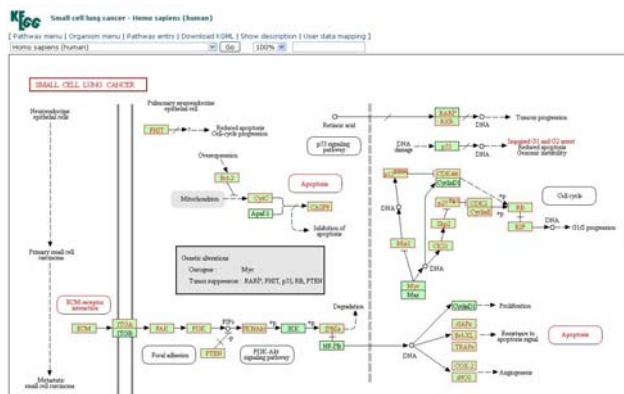


Fig 3: COX-2 Pathway of Lung cancer

Structure and Function of COX-2 in human

Cytochrome c oxidase is an oligomeric enzymatic complex which is a component of the respiratory chain and is involved

in the transfer of electrons from cytochrome c to oxygen. In eukaryotes this enzyme complex is located in the mitochondrial inner membrane; in aerobic prokaryotes it is found in the plasma membrane. The enzyme complex consists of 3-4 subunits (prokaryotes) to up to 13 polypeptides (mammals). In Leigh's disease, there may be an abnormality or deficiency of cytochrome oxidase.

Subunit 2 (COII) transfers the electrons from cytochrome c to the catalytic subunit 1. It contains two adjacent transmembrane regions in its N-terminus and the major part of the protein is exposed to the periplasmic or to the mitochondrial intermembrane space, respectively. COII provides the substrate-binding site and contains a copper centre called Cu (A), probably the primary acceptor in cytochrome c oxidase. An exception is the corresponding subunit of the cbb3-type oxidase which lacks the copper A redox-centre. Several bacterial COII have a C-terminal extension that contains a covalently bound haem c.

The N-terminal domain of cytochrome C oxidase contains two transmembrane alpha-helices.

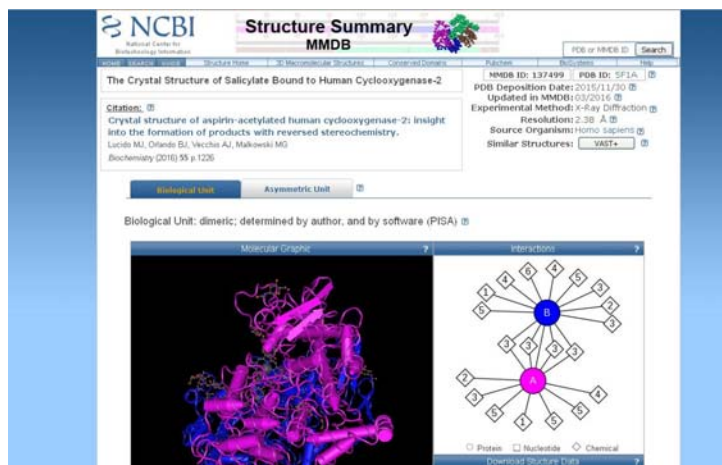


Fig 4: Molecular Summary of COX-2 in Protein database

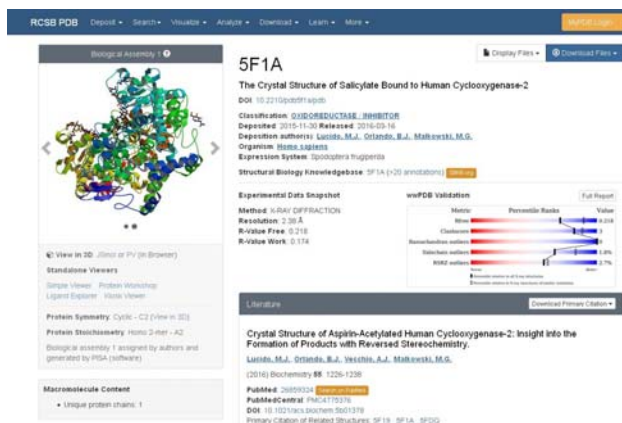


Fig 5: Protein Database Validation of COX-2

Results

Extensive study shows that, by dint of execution of recent multidimensional analytical process as well as with the acquisition of knowledge, it is now possible to predict in advance and more accurately for many more experiments which in turn helps the both 'in vivo' and 'in vitro studies'. Paying regards to that, we can say that IL-8 and VEGF were

detected by ELISA in conditioned medium after 48 h incubation in a panel of lung cancer cell lines. In all three tested NSCLC cell lines, IL-8 production was significantly ($p < 0.001$) higher than VEGF production. In contrast, VEGF production was significantly ($p < 0.001$) higher than IL-8 production in all five tested SCLC cell lines. NSCLC cell lines consistently produced higher levels of IL-8 (above 5.6 ng/ml/ 10^6 cells) than SCLC cell lines (under 0.9 ng/ml/ 10^6 cells). However, there was no significant difference in VEGF levels between NSCLC (ranging from 3.7 to 6.3 ng/ml/ 10^6 cells) and SCLC (ranging from 4.0 to 11.2 ng/ml/ 10^6 cells) cell lines.

Depending upon the analysis, we can clearly say that, Effects of COX-2 inhibition on production of VEGF and IL-8, NS-398 is a potent and specific COX-2 inhibitor [22]. To confirm that NS-398 effectively targeted COX-2 in the NSCLC cell lines, we measured PGE₂ production by ELISA and found that it was reduced in a dose-dependent fashion by NS-398 at 48 h treatment in A549. PGE₂ was reduced to 390 pg/ml ($p < 0.05$) by NS-398 at 2 μ M and to 105 pg/ml ($p < 0.01$) by NS-398 at 20 μ M from 937 pg/ml, respectively. At 24 h treatment, PGE₂ was significantly ($p < 0.01$) reduced by NS-398 (20 μ M) at 24 h in all three tested cell lines (Figure 3C). COX-2 expression was not changed by NS-398 at up to 2 μ M, and slightly

reduced by NS-398 at 20 μ M as shown by Western blotting, whereas COX-1 was not affected.

Discussion

Several studies have shown that COX-2, VEGF and IL-8 are over expressed in lung cancer compared to normal bronchial epithelium. Most studies have focused on NSCLC since clinical specimens of SCLC are difficult to obtain for research because SCLC patients are rarely operated on. Tas *et al* [23, 24] recently reported that serum VEGF levels were significantly higher in lung cancer patients than healthy controls. Mean serum VEGF levels appeared to be slightly higher in SCLC (1350 pg/ml, range 170 – 3810 pg/ml, n = 34) than NSCLC patients (402 pg/ml, range 121 – 1800 pg/ml, n = 52), but this was not tested statistically.

Here, we have compared and contrasted SCLC and NSCLC using a panel of cell lines. Basal expression of VEGF, IL-8, COX-1, COX-2 and PGE₂ was determined in three NSCLC and five SCLC cell lines. VEGF was produced at similar levels in all cell lines. NSCLC cell lines produced much more IL-8 than SCLC cell lines. COX-1 was expressed at similar levels in all cell lines, whereas COX-2 was only expressed in NSCLC cell lines, albeit at low levels in H460. Interestingly, high levels of PGE₂ were not only produced in MOR/P and A549 cell lines as a result of strong COX-2 expression, but also in H460 cell line in which COX-2 expression was weak. This could result from reduced catabolism of PGE₂ due to low levels of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a primary enzyme responsible for PGE₂ metabolism [25]. Previous studies showed that NSCLC cells expressed COX-2 protein [17, 26]. However, one study showed that a subset of 20% of SCLC patients (11 out of 54) expressed COX-2 [27]. These data, together with our results and those of Pold *et al* [20], appear to suggest an association between COX-2 expression and IL-8 production in lung cancer cells. In contrast, VEGF production did not seem to be solely related to COX-2 expression as both COX-2 positive and negative cell lines produced similar levels of VEGF in our study.

COX-2 expression has previously been associated with VEGF in patients with NSCLC [19, 20]. Two reports from Dubinett's lab showed that expressions of IL-8 and VEGF were enhanced in NSCLC cell lines transfected with retroviral COX-2 vector [20, 28]. The aim of this study was to exam the causal relationship between COX-2 and IL-8 or VEGF in lung cancer cell through inhibition of endogenous COX-2 using either a potent and specific COX-2 inhibitor, NS398 or COX-2 siRNA. NSCLC cell lines were incubated with NS-398 for 24 h and 48 h. NS-398 exerts its effects by directly inhibits COX-2 activity. We demonstrated that COX-2 activity was inhibited by NS-398 as evidence of reduced production of PGE₂ in these cell lines. We decided to use a maximum concentration of 20 μ M in this study because COX-2 inhibition had been demonstrated and cell growth was not significantly inhibited in NSCLC cell lines as shown in at this level. NS-398 has been shown to induce 16% apoptosis in H460 cells at 300 μ M in another study [29]. Unexpectedly, IL-8 production was not altered after treatment with NS-398 in these cell lines. However, VEGF was significantly decreased by NS-398 in A549 and MOR/P cell lines, but not in H460. Both of A549 and MOR/P cell lines expressed high levels of COX-2 whereas H460 expressed very low level of COX-2 in our experiments. In some reports, H460 was considered as COX-2-negative cell line [29]. These results suggested that VEGF production was independent of COX-2 in H460 and other COX-2-negative cells such as SCLC cell

lines. We further confirm the direct involvement of COX-2 on VEGF production using COX-2 siRNA, and similar results were found. VEGF was significantly reduced in both of A549 and MOR/P, but not in H460. IL-8 was not affected following treatment with COX-2 siRNA in all tested NSCLC cell lines. These results suggest that VEGF is, at least partly, COX-2 dependent in COX-2-expressing NSCLC cells such as A549 and MOR/P. This finding is in agreement with other findings [19, 20, 28, 30]. However, VEGF production is clearly not depend on COX-2 status in other lung cancer cells such as SCLC cells, which expressing no COX-2, and some NSCLC cells including H460, which expressing very low level of COX-2. Our results also suggest that IL-8 is COX-2 independent in lung cancer cells. This finding seems to contradict others [28]. The different findings may be due to the different experiments systems (e.g. to introduce ectopic overexpression of COX-2 or to knock down endogenous COX-2 by siRNA) employed in these studies. Interestingly, Raut *et al* [31] reported that blocking COX-2 production by NS-398 in pancreatic cancer cell lines did not affect VEGF, bFGF and IL-8 production, but Singh *et al* [32] found that NS-398 downregulated IL-8 by 30% in a COX-2 transfected MDA-231 breast cancer cell line. These contradictory findings suggest that the relationship between COX-2, VEGF and IL-8 is complex. More studies are required to establish the causal relationship between COX-2 and IL-8 and VEGF in lung cancer. COX-2 inhibitors have been shown to reduce angiogenesis and metastasis in lung cancer [21], our results suggest that anti-angiogenic effects of COX-2 inhibitors might be, at least partly, mediated by inhibition of VEGF, but not IL-8. As IL-8 seems to be independent of COX-2, therefore, there is a strong rationale for combining treatments with inhibitors of COX-2 and IL-8 in NSCLC.

Conclusion

Our results suggest that NSCLC cells produce much more IL-8 than SCLC cells whereas both NSCLC and SCLC cells produce similar levels of VEGF. COX-2 is only expressed in NSCLC cells, but not in SCLC cells. VEGF can be produced in lung cancer cells regardless of COX-2 expression. However, VEGF production is, at least partly, COX-2 dependent in NSCLC cells expressing COX-2. In contrast, IL-8 production is COX-2 independent in both NSCLC and SCLC cells. We speculate that combined targeting of COX-2 and IL-8 may be useful in the treatment of patients with NSCLC and targeting VEGF may be useful in the treatment of patients with SCLC.

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