SPANDIDOS PUBLICATIONS

Specific components of prostanoid-signaling pathways are present in non-small cell lung cancer cells

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Abstract. In the present study, we measured prostanoid synthesis and the expression of genes associated with prostanoid signaling in human non-small cell lung carcinoma (NSCLC) cell lines and in primary human tumors. Consistent with the proposed growth promoting role of PGE_2, we found that NSCLC cell lines frequently co-expressed the genes encoding cyclooxygenase-2 and the prostaglandin E_2 (PGE_2) receptors EP1, 2 and 4 concomitant with the synthesis of PGE_2. In contrast, NSCLC cells did not synthesize appreciable amounts of prostaglandin I_2 (PGI_2, prostacyclin), lacked PGI_2 synthase (PGIS) and did not express the gene coding for the PGI_2 receptor IP at detectable levels. In agreement with this finding, *PGIS* mRNA levels were dramatically diminished in primary human tumor samples as compared to matched normal lung tissue. Finally, thromboxane A_2 (TxA_2) was synthesized in NSCLC cell lines, but transcription of the gene coding for the TxA_2 receptor TP was not observed in these cells. In marked contrast, lung fibroblasts synthesized all three prostanoids and their receptors at high levels. While the observed expression patterns were consistent with the existence of autocrine/paracrine PGE_2 signaling loops in NSCLC cells, PGI_2- and TxA_2-mediated signals may play a role in tumor stroma cells.

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Introduction

The cyclooxygenases (Cox-1, Cox-2) are rate-limiting enzymes in the synthesis of all prostanoids from arachidonic acid (1). Research in the past decade has clearly established Cox-2 as a crucial player in tumor growth and progression (2-4). Cyclooxygenases catalyze a two-step reaction that converts arachidonic acid to prostaglandin H_2 (PGH_2), which in turn serves as the precursor for the synthesis of the biologically active prostanoids PGD_2, PGE_2, PGF_2, PGI_2 (prostacyclin) and thromboxane A_2 by specific prostanoid synthases (1). The prostanoids act as agonists of G-protein-coupled membrane receptors, termed DP, EP1 through EP4, FP, IP and TP, which in turn trigger multiple second messenger generating systems (5,6). Several specific components of this prostanoid signaling network have been associated with oncogenesis, in particular PGE_2 and its membrane receptors EP2, EP3 and EP4 (6). These conclusions are based on results obtained with knock-out mice as well as selective agonists or inhibitors (7-13). On the other hand, the role of PGI_2 is controversial, as both tumor growth-promoting and inhibitory effects have been described (14-16). The role of other prostanoids is largely unclear, even though TxA_2 has been reported to enhance tumor growth in mice (17). In human non-small cell lung carcinoma (NSCLC), expression of Cox-2 and prostaglandin E_2 synthase (PGES) has been demonstrated in several studies (18), and there is strong evidence for a tumor-promoting role of Cox-2 and most likely PGE_2 on these tumors (19-24). Thromboxane A_2 synthase (TxAS) and prostaglandin I_2 synthase (PGIS) expression in NSCLCs has been analyzed, but this study relied entirely on immunohistochemistry (25). We therefore sought to perform a systematic analysis of prostanoid synthesis and gene expression associated with prostanoid signaling in human NSCLCs.

Materials and methods

Cell culture. NSCLC cell lines were obtained from K. Havemann (Marburg). LLC1, WI38 and HEL cells were

Table I. The primers and annealing conditions.

Human genes	Sequence 5'→3'	Length (nt)	Product (bp)	Annealing temperature (˚C)
ß-actin	GATGATGATATCGCCGCGCTCGTCGTC	24	800	53-62
	GTGCCTCAGGGCAGCGGACCGCTCA	25		
COX-1	GGTGGGGTGTTCTTCTTG	18	1125	58
	TCCCTCAACCCCTCATCTT	19		
COX-2	CCTTCTCCAACCTCTCCTAC	20	748	58
	AGGGGGTGCCAGTGATAGAG	20		
mPGES	CACGCTGCTGGTCATCAAGA	20	330	61
	TAGGTCACGGAGCGGATGG	19		
PGIS	AGGAGAAGCACGGTGACATC	20	386	58
(RT-PCR, qPCR)	GCAGCGCCTCAATTCCGTAA	20		
TxAS	GGTGCCCTGATGTCTGCTTT	20	686	56
	TCTTGGCAGTCAGGGTTGGT	20		
EP1	CCTCGTCCGCCTCGTCCATC	20	317	63
	CGGGGGCAAGAGGCGAAGCA	20		
EP2	GCTGCCGCTGCTGGACTATG	20	328	63
	CAAGGAGCAGACGGCGAAGG	20		
EP3	CCCGCCTCAACCACTCCTAC	20	669	63
	CCAGGAAGGCAAAGGCAGAG	20		
EP4	CCTCCTGAGAAAGACAGTGCT	21	366	60
	AAGACACTCTCTGAGTCCT	19		
IP	AAATAACCAGTGGCCTGGC	19	161	53
	TTGGGCCTCCTAAGTGGAC	19		
TP	TTCGCCGCCTCCTTCTGC	18	606	64
	CGCCTCCTGCCCGTGGTA	18		
L27	TGATGGCACCTCAGATCGC	19	169	58
(qPCR)	AGAGTACCTTGTGGGCATTAGG	22		

purchased from the ATCC. NIH3T3 cells were provided by D. Lowy (NIH), and Line-1 (26) and other mouse lung adenoma lines by G. Stoner (Ohio State University). All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Human bronchial epithelial cells (HBEPCs) were purchased from Promocell (Heidelberg, Germany) and cultured as suggested by the supplier. Cells were maintained at 37˚C with 5% CO_2 in a humidified incubator.

RNA isolation. RNA was isolated by using the RNeasy™ kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Qia shredders (Qiagen) were used to break down genomic DNA of lysed cells.

Reverse transcriptase (RT-PCR) and real-time PCR (qPCR). cDNA was synthesized using 1 μg of RNA, oligo(dT) primers and reverse transcriptase according to the manufacturer's protocol (Roche Diagnostics). RT-PCR was performed with Platinum Taq polymerase (Invitrogen, Karlsruhe, Germany). qPCR reactions were carried out with the Absolute QPCR SYBR-Green mix (Abgene, Hamburg, Germany) at a primer concentration of 0.2 μM in an Mx3000P Real-time PCR system (Stratagene, La Jolla, CA) for 45 cycles. Primers and annealing conditions were as described in Table I. For qPCR experiments, *L27* was used as the normalizer. Results were expressed as Ct values corrected for differences in *L27* expression (average Ct value for *L27*=20).

Prostanoid analysis by GC/MS/MS analysis. For the determination of prostanoid release, 10^6 cells were cultured on 6-cm tissue culture dishes in 5 ml of fresh DMEM + 10% fetal bovine serum for 24 h. Samples were prepared as described (27) with minor modifications. Briefly, cell culture supernatants were spiked with ~10 ng of deuterated internal standards, and the solvent was removed. The methoxime was obtained through reaction with an O-methylhydroxylamine hydrochloride-acetate buffer. After acidification to pH 3.5, prostanoid derivatives were extracted, and the pentafluorobenzylesters were formed. Samples were purified by thin-layer chromatography, and two broad zones with R_v 0.03-0.39 and 0.4-0.8 were eluted. After withdrawal of the organic

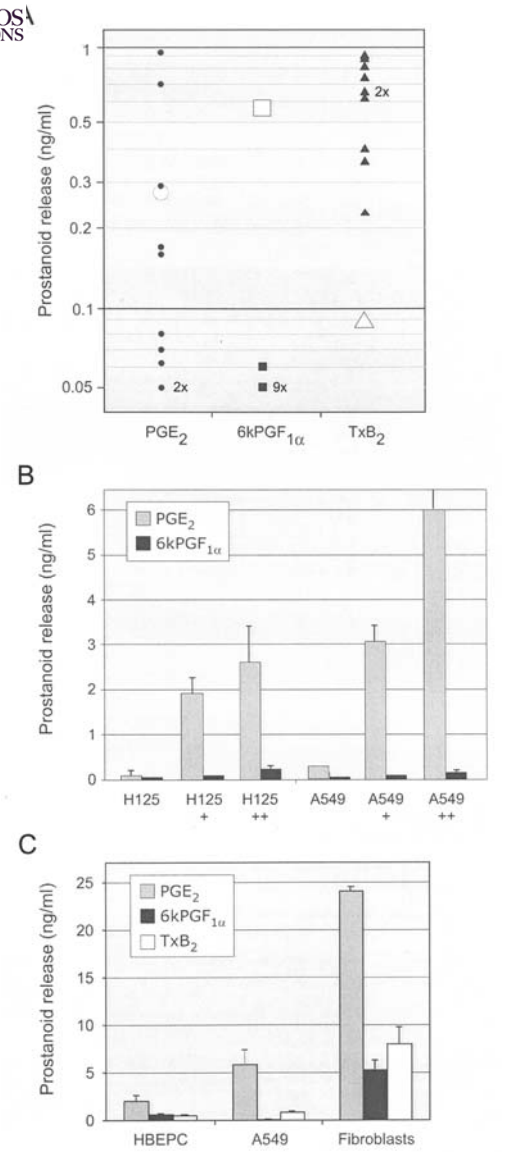

Figure 1. Prostanoid release by human NSCLC cell lines. (A) PGE_2, 6-keto-$PGF_{1\alpha}$ and TxB_2 levels in the medium of the 10 different human NSCLC cell lines (filled marks) and normal HBEPCs (open marks) were determined by GC-MS analysis. Data points for PGE_2 in NSCLC cell lines (solid circles) from top to bottom (values in ng/ml): H2073 (0.92), A549 (0.7), H596 (0.3), H322 (0.17), H23 (0.15), H125 (0.08), H2347 (0.07), H1373 (0.06), H522 (0.05), H1651 and (0.05). Data points for 6-keto-$PGF_{1\alpha}$ (solid squares) from top to bottom: H2073 (0.06) and all others (<0.05). Data points for TxB_2 (solid triangles) from top to bottom: H125 (0.90), H23 (0.88), H596 (0.82), H522 (0.74), H322 (0.66), H2347 (0.65), H1373 (0.62), A549 (0.40), H2073 (0.36), H1651 (0.23). The graphs show the concentration of the indicated prostanoids after culturing 10^6 cells in 5 ml of medium for 24 h. (B) PGE_2 and 6-keto-$PGF_{1\alpha}$ released by human NSCLC cells in the presence of 10 μM arachadonic acid (+) or 10 μM arachadonic acid + 100 nM TPA + 3 μM of the calcium ionophore A23187 (++). (C) Comparison of prostanoid released by normal HBEPCs, A549 NSCLC cells and HEL fibroblasts in the presence of 10 μM arachadonic acid.

layers, trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl)-trifluoroacetamide and thereafter subjected to GC/MS/MS analysis on a Finnigan (Thermo Electron Corp., Dreieich, Germany) MAT TSQ700 GC/MS/MS equipped with a Varian (Palo Alto, CA) 3400 gas chromatograph and a CTC A200S (27).

Immunoblot analysis. Expression of PGIS protein was analyzed by immunoblot analysis using a rabbit polyclonal anti-PGIS antibody as previously described (28). Briefly, 20 μg of total cell lysate was separated on 10% SDS-PAGE, blotted onto nitrocellulose, and probed with the rabbit polyclonal anti-PGIS antibody (diluted 1:500).

Results

Prostanoid synthesis in human NSCLC cells and fibroblasts. We first measured the synthesis of prostanoids by 10 different human NSCLC cell lines. PGE_2 was consistently found in the growth medium of all 10 cell lines tested, albeit at variable levels, with the highest values found for A549, H2073, H596 and H322 cells (Fig. 1A). In contrast, the concentration of 6-keto-$PGF_{1\alpha}$ (a direct metabolite of, and marker for, the unstable PGI_2) was very low or undetectable, and in all cases clearly below the biosynthetic rate of normal human bronchial epithelial cells (HBEPCs). In contrast, TxB_2, a direct metabolite of TxA_2, was released by all NSCLC cell lines at moderate but significantly higher levels compared to HBEPCs. PGD_2 was also determined but was very low in all cases including HBEPCs (data not shown). To determine whether the lack of PGI_2 synthesis was due to rate-limiting substrate concentrations, we also performed the experiment in the presence of 10 μM arachidonic acid (Fig. 1B). However, in contrast to PGE_2, no significant effect on the release of 6-keto-$PGF_{1\alpha}$ was observed. Likewise, the simultaneous stimulation of Cox-2 by the phorbol ester TPA and a calcium ionophore further enhanced PGE_2 synthesis in the absence of a significant increase in the release of 6-keto-$PGF_{1\alpha}$ (Fig. 1B). We also determined prostanoid synthesis in human fibroblasts (HEL; Fig. 1C). As expected, these cells released high levels of PGE_2, 6-keto-$PGF_{1\alpha}$ and TxB_2.

Expression of genes encoding enzymes of prostanoid synthesis and prostanoid receptors. Next, we investigated the expression of the *Cox-1* and *Cox-2* genes in NSCLC cells in comparison to WI38 lung fibroblasts (Fig. 2A). As expected, *Cox-2* was the predominantly expressed subtype observed in all NSCLC cells but H2073 strongly expressed both *Cox-1* and *Cox-2*. The levels of *Cox-2* mRNA in NSCLC cell lines showed a good correlation with the rate of PGE_2 biosynthesis (Fig. 1A). Consistent with the lack of PGI_2 synthesis, many of the NSCLC cell lines did not express *PGIS* at detectable levels, and in those cases where *PGIS* mRNA was found, PGIS protein was extremely low or undetectable (Fig. 2B). This suggests that NSCLC cells down-regulate the synthesis of PGI_2 at multiple levels, including transcriptional and translational mechanism(s). Analysis of the mRNA expression profiles for all known prostanoid membrane receptors showed a high and almost uniform expression for *EP1*, *EP2* and *EP4*, while expression of *EP3*, *IP* and *TP* was basically undetectable in the vast majority of the NSCLC cell lines (Fig. 2C). In contrast, *EP1*, *EP2*, *EP4*, *IP* and *TP* expression was clearly detectable in human fibroblasts (Fig. 2C).

Prostanoid synthesis in mouse lung tumor cells and fibroblasts. Next, we studied the synthesis of prostanoids by

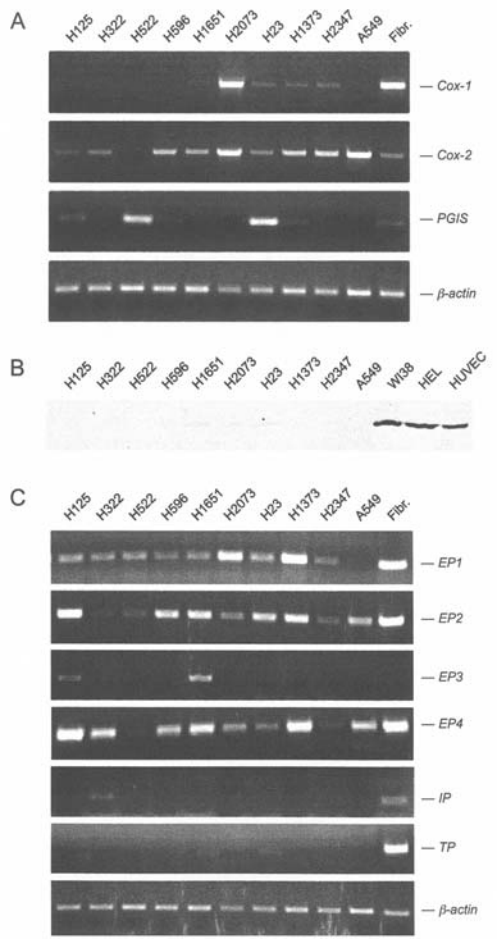

Figure 2. Expression of genes involved in prostanoid biosynthesis and signaling by human NSCLC cell lines. (A) Expression of *Cox-1*, *Cox-2* and *PGIS* mRNA in human NSCLC cells and WI38 fibroblasts. RNA was extracted from normally cycling cells, and mRNA levels were determined by RT-PCR. ß-actin was included as the standard. (B) Immunoblot analysis of PGIS levels in human NSCLC cell lines, WI38 and HEL fibroblasts and HUVEC. Equal loading was verified by staining for ß-actin (not shown). (C) Expression of genes coding for prostanoid membrane receptors in human NSCLC cell lines and HEL fibroblasts. RNA was analyzed as in panel D for mRNA levels of *EP1*, *EP2*, *EP3*, *EP4*, *IP* and *TP*.

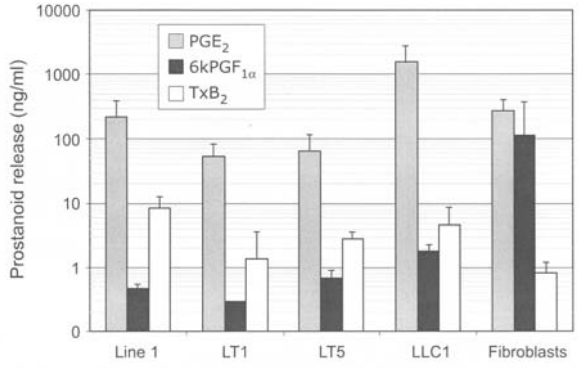

Figure 3. Prostanoid release by mouse lung tumor cell lines and NIH3T3 fibroblasts. The data represent the concentrations of PGE_2, 6-keto-$PGF_{1\alpha}$ and TxB_2 after culturing 10^6 cells in 5 ml of medium for 24 h. Bars represent average values, error bars show the standard deviation.

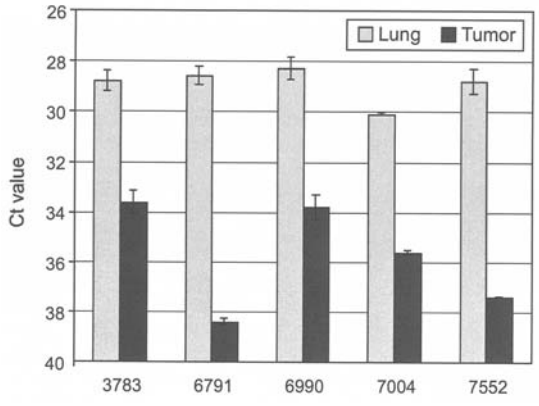

Figure 4. *PGIS* expression in primary human NSCLCs. *PGIS* mRNA levels in matched samples of primary human NSCLCs and normal lung tissue from the same patient were analyzed by qPCR. Numbers indicate anonymized patient IDs. Bar graphs in all panels represent average Ct values (2-5 measurements) and error bars show the standard deviation. *L27* was used as the normalizer for all samples.

cell lines derived from mouse lung adenoma and adenocarcinoma (Fig. 3). All four cell lines showed a high rate of PGE_2 synthesis, failed to release 6-keto-$PGF_{1\alpha}$ at significant levels and showed low but readily detectable levels of TxB_2 release. These data are in agreement with our observation made with human lung cancer cell lines, suggesting that these findings are of general relevance to lung adenocarcinomas.

Gene expression patterns in primary human NSCLCs. Finally, we analyzed the expression of *PGIS* in five matched pairs of human NSCLCs and normal lung tissue to exclude that the lack of PGI_2 synthesis observed in NSCLC cell lines is a cell culture artifact. As can be seen in Fig. 4, expression of *PGIS* mRNA was dramatically reduced in all tumor tissue samples compared to the normal lung (5-10 Ct values, corresponding to an ~30- to 1000-fold difference). This result is fully compatible with our study of established lung tumor cell lines, and is also in agreement with published immunohistochemical observations (25). We also measured *IP* expression, which was equally low in all tumor and lung tissue samples analyzed (Ct ≥31; data not shown). This finding is also in agreement with our cell culture data.

Discussion

A vast body of evidence suggests that the tumor-promoting effects of Cox-2 are attributable to a major extent to overproduction of PGE_2 and the ensuing stimulation of PGE_2 membrane receptors (7,8,10,11,9,29-32). Particularly strong evidence derived from mouse models exists for EP2 and EP4 in gastrointestinal tumors (29,30) and mammary carcinoma (32). Our findings obtained with human NSCLCs are consistent with this picture and suggest that autocrine/paracrine PGE_2 signaling might be of high relevance for these cells. In contrast, TxA_2 is synthesized by NSCLCs at appreciable amounts, but NSCLC cells fail to express the gene encoding its receptor, *TP*. On the other hand, fibroblasts strongly express *TP* and synthesize TxA_2, which would be compatible with a role in the growth supporting stroma. Strikingly,

cells do not synthesize PGI_2 due to a lack of PGIS t express the PGI_2 receptor gene, *IP*. These findings indicate that PGI_2-driven signaling pathways are not essential for NSCLC cells, and may even point to a potentially negative effect on tumor growth. This hypothesis is indeed supported by a previous study that showed suppression of lung adenoma growth by the pneumocyte-directed expression of a *PGIS* transgene in mice (15).

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