Increased Gene Expression of the ABCC5 Transporter without Distinct Changes in the Expression of PDE5 in Human Cervical Cancer Cells during Growth

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Abstract. *Carcinoma of the uterine cervix represents the second most frequent female malignancy worldwide, but few biochemical tumour markers have been implemented into clinical practice. Elevated extracellular guanosine 3', 5'-cyclic monophosphate (cGMP) levels have been reported to be a sensitive, early and reliable marker for screening relapse in carcinoma of the uterine cervix. The mechanism behind this observation remains unknown. The possibility exists that the cancer cells develop resistance to the antiproliferative effect of high intracellular cGMP levels. The enhanced cGMP expression may originate from either an increase in cellular export capacity by increased expression of member 5 in subfamily C of ATP-Binding-Cassette transporters (ABCC5), or increased substrate (cGMP) levels for this pump. The latter situation occurs with increased expression of inducible nitric oxide synthase (iNOS) and/or soluble guanylyl cyclase (sGC) and/or reduced expression of member 5 of the cyclic nucleotide phosphodiesterases (PDE5). Four transformed human cell lines derived from carcinomas of the uterine cervix (C-4 I, C-33 A, SiHa and ME-180 cells) and one non-transformed human cell line (WI-38) were included in the study in order to unveil which biokinetic components are involved. The expressions of iNOS, sGC, PDE5 and ABCC5 in the initial and final phase of the exponential growth curve were compared. Assuming that the WI-38 control cells mimic the situation in a normal tissue, iNOS remains un-expressed during proliferation, and the expression of sGC is low but shows a clear increase during exponential growth. PDE5 is highly expressed and increases (≈130%) during growth whereas ABCC5 exhibited low to moderate expression, with a moderate increase (≈40%) during growth. The malignant cells exhibited moderate ABCC5 expression with a distinct increase during exponential growth, whereas PDE5 expression remained virtually unchanged. Dysregulation of the cGMP biokinetics in growing malignant cells may account for the elevation of extracellular cGMP observed in patients with carcinoma of the uterine cervix.*

Carcinoma of the uterine cervix represents the second most frequent female malignancy worldwide (1). Great effort has been made in the search for reliable tools to predict prognosis and evaluate the effectiveness of treatment for this type of gynaecological cancer. Very few biochemical tumour markers have, so far, shown sufficient robustness to deserve implementation into clinical practice. Several serological markers including carcinoembryonal antigen (CEA), tissue polypeptide antigen (TPA), cancer antigen CA-125, amino-terminal propeptide of type III procollagen (PIIINP) and squamous cell carcinoma antigen (SCC-Ag) have been proposed, but their significance is still controversial (2, 3). However, an elevated extracellular level of guanosine 3', 5'-cyclic monophosphate (cGMP) has been reported by many authors to be a sensitive, early and reliable marker for relapse screening in gynaecological malignancies such as ovarian cancer (4-7) and carcinoma of the uterine cervix (8, 9). Elevated urinary levels of cGMP were detected three months after primary treatment in patients with gynaecological cancer who experienced disease relapse within the respective observation periods of 3.25 years (8) and 10 years (9).

The increased plasma and urinary levels of cGMP in a variety of human cancers reflect an increased rate of entry of the nucleotide into the plasma *i.e.* cellular efflux, and not enhanced clearance (10). Increased soluble guanylyl cyclase

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(sGC) activity or reduced cyclic nucleotide phosphodiesterase (cnPDE) activity will, alone and in combination, increase the cellular cGMP efflux. However, even without a change in substrate concentration, an increased expression of member 5 in subfamily C of ATP-Binding Cassette transporters (ABCC5), also known as multiresistance-associated protein type 5 (MRP5), will produce elevated extracellular levels of cGMP. ABCC5 is an ATPase driven cGMP pump which is responsible for the export of cellular cGMP (11). In a previous study on tissue samples from patients with carcinoma of the uterine cervix, we used immunohistochemistry to relate the expression of inducible nitric oxide synthase (iNOS), sGC and member 5 of the cnPDE family (PDE5) to prognosis (12). To further illuminate these biological mechanisms, four different human cervical carcinoma cell lines are used here, as a model system. In the current *in vitro* study, we investigated the mRNA expression of different molecular components of the cGMP pathway (iNOS, sGC, PDE5 and ABCC5) by utilizing quantitative polymerase chain reaction (qPCR) technology.

Materials and Methods

Cell lines. Human cervical squamous carcinoma cell lines C-4 I (13), C-33 A (14), SiHa (15) and ME-180 (16) and control cell line WI-38 (17) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in RPMI-1640 with 10% (v/v) foetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), at 37˚C in triplicates. Cell density was monitored daily and the cells were harvested by exposure to trypsin-EDTA in the initial and terminal period of the log phase. The cell viability was determined with trypan-blue exclusion. The harvested cells were washed with PBS and parallel samples were harvested from each cell line.

RNA isolation and cDNA synthesis. RNA was isolated using RNAeasy Mini Kit (Qiagen Nordic, Sollentuna, Sweden). Total RNA (1 μg) was reverse transcribed in a 20 μl reaction mixture using random hexamer primers and the Bio Primescript II 1st strand cDNA synthesis kit (Takara Bio Inc, Shiga, Japan) according to the manufacturer's protocol. The resulting cDNA was diluted 3-fold. The cDNA used to generate the standard curve, was reverse-transcribed from 2.5 μg QPCR reference RNA (1 μg/μl) (Agilent Technologies Inc. Santa Clara, CA, USA) in a 20 μl reaction mixture. The standard cDNA was diluted 3-fold and then serially diluted in triplicate 5-fold dilutions.

Quantitative polymerase chain reaction and relative quantification of gene expression. qPCR analysis was performed for the target genes *iNOS,* α-subunits of *sGC, PDE5A, ABCC5* and the endogenous reference genes glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* and hypoxanthine phosphoribosyltransferase *(HPRT),* using the Solaris qPCR Gene Expression Assays and the Solaris qPCR Gene Expression Mastermix including ROX (ThermoFisher Scientific, Waltham, MA, USA). The assay was run with 5 μl cDNA in a 20 μl total reaction volume on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Non-template controls were assayed in parallel. The amplification conditions were 95˚C for 15 min, 95˚C for 15 s for 40 cycles, and 60˚C for 60 s. A standard curve was computed for each target and reference gene separately by plotting the known concentration of the serially diluted standard cDNA against the CT value. The relative concentration of the target genes in each sample was calculated from the standard curve.

Table I. *Expression of inducible nitric oxide synthase (iNOS) was determined, as described in the Material and Methods, and the expression was calculated in relation to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT). The results are shown as the mean value±SD from three experiments.*

	Cells				
iNOS	WI-38	C-4 I	C-33 A	SiHa	ME-180
GAPDH					
Initial log-phase	UD	UD	2.5±1.2	2.1±0.8	0.0127±0.0077
Terminal log-phase	UD	UD	0.87±0.13	1.3±1.1	0.0225±0.0100
HPRT					
Initial log-phase	UD	UD	1.4±0.6	3.1±1.3	0.0130±0.0079
Terminal log-phase	UD	UD	0.87±0.12	1.4±1.1	0.0247±0.0110

UD, Gene expression undetectable.

Results

Expression of iNOS. In a recent study (12) we demonstrated the protein expression of iNOS in carcinomas of the uterine cervix as being a favourable prognostic factor. This observation was followed-up by characterization of *iNOS* gene expression in the initial and final phases of exponential human cervical cell growth. In the present study, *iNOS* gene expression varied considerably (Table I) and there was no consistent change during growth (Figure 1). Expression was undetectable in WI-38 control cells and C-4 I cells, and detectable but with a very low signal in ME-180 cells. On the other hand, the expression was much higher in C-33 A and SiHa cells (Table I), but was found to decrease during growth (Figure 1).

Expression of sGC α subunits. In human sGC, two α and two β subunits have been identified (18). Heterodimer formation is necessary for catalytic function and responsiveness to nitric oxide (NO). The α_1/β_1 sGC heterodimer is most abundant, but heterodimers containing α_2 and β_2 subunits and splice variants also exist (18-20). In the present study, gene expression of *sGC* subunits α_1 and α_2 (Figure 2) and their change in their expression during growth (Table II), varied considerably. The expression of subunit α_1 in WI-38 control cells was low, but increased markedly during growth. Subunit α_2 was not expressed. In C-4 I cells, the α_1 subunit exhibited considerably higher expression, which increased moderately during growth, while subunit α_2 was not

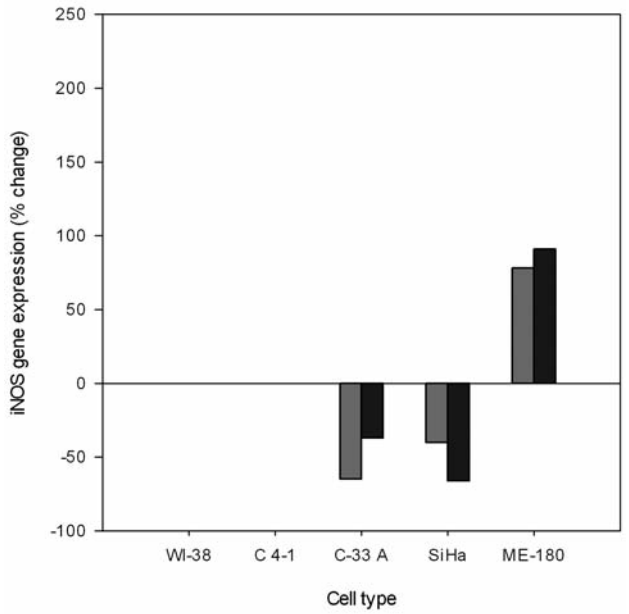

Figure 1. *Changes in expression of inducible nitric oxide synthase (iNOS) during exponential growth of normal human fibroblasts (WI-38) and human cell lines from cancer of the uterine cervix (C-4 I, C-33 A, SiHa and ME-180). iNOS expression was calculated in relation to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (red) and hypoxanthine phosphoribosyltransferase (HPRT) (black). The results are presented as the mean value of three growth curves.*

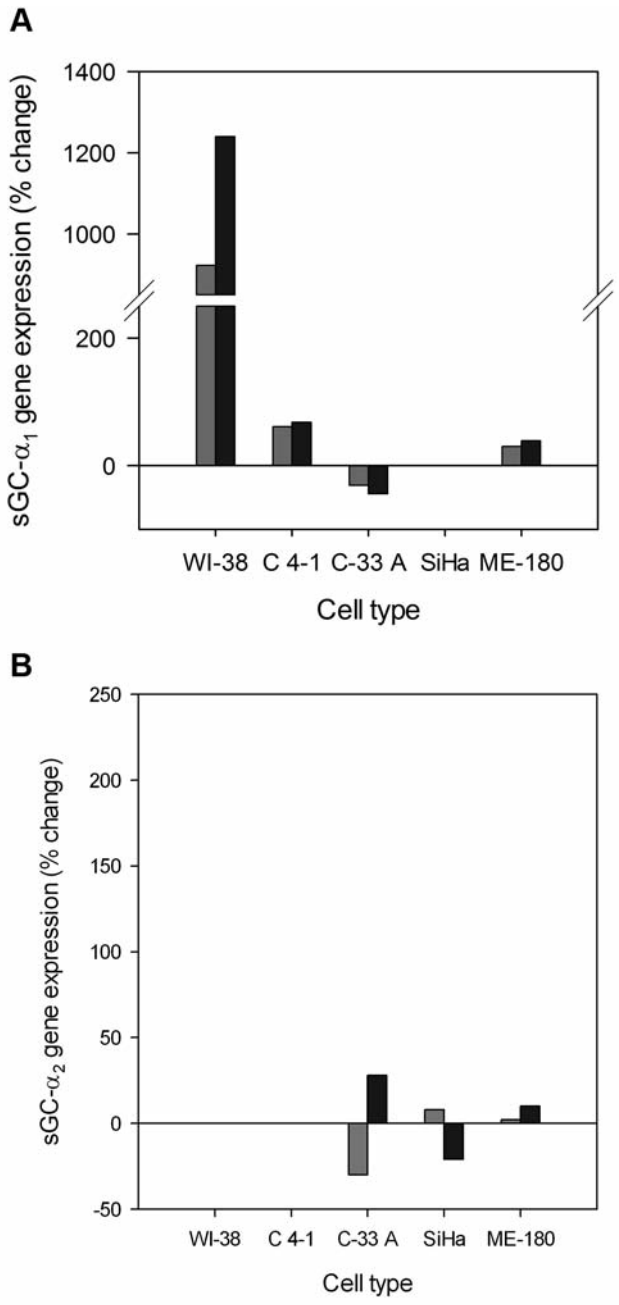

Figure 2. *Changes in expression of soluble guanylyl cyclase (sGC) subunits α_1 (A) and α_2 (B) during exponential growth. The expression of sGC subunits was calculated in relation to that of GAPDH (red) and HPRT (black). The results are presented as the mean value of three growth curves. Notice the difference in ordinate scaling.*

expressed. Expression of both subunits was low in C-33 A cells and tended to decrease, or showed no clear change during growth. Subunit α_1 was not expressed in SiHa cells, but subunit α_2 was highly expressed, without showing any consistent growth-associated changes. Expression of subunit α_1 was hardly detectable in ME-180 cells; subunit α_2 was moderately expressed and its expression did not change during growth.

Expression of PDE5. Cyclic nucleotide phosphodiesterases are important for termination of the biological signalling by cGMP and cAMP. PDE5 is specific for cGMP (21) and is widely distributed in human tissues (22). Table III shows that WI-38 control cells differ distinctly from the transformed cells, by their much higher expression of PDE5. Its gene expression increased during growth in WI-38, C-4 I and ME-180 cells, but decreased in C-33 A and SiHa cells (Figure 3). There was no consistent change in expression among the transformed cell types during growth and expression was generally much lower (Figure 3).

Expression of ABCC5. At physiological levels of cGMP, ABCC5 is mainly responsible for the cellular extrusion of this molecule (23). The most prominent feature of the *ABCC5* gene expression was its consistent increase during the exponential growth in both normal and transformed cell lines (Figure 4). Table IV shows that C-33A cells exhibited low expression, whereas WI-38 control cells exhibited low to moderate expression, and C-4 I, SiHa and ME-180 cells all had distinct, but moderate *ABCC5* gene expression.

Table II. *Expression of soluble guanylyl cyclase (sGC) subunits α_1 and α_2 was determined as described in the Material and Methods and the expression was calculated in relation to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT). The results are shown as the mean value±SD from three experiments.*

	Cells				
sGC1α_1	WI-38	C-4 I	C-33 A	SiHa	ME-180
GAPDH					
Initial log-phase	0.01±0.02	3.0±0.7	0.0160±0.004	UD	0.0008±0.0001
Terminal log-phase	0.10±0.04	4.8±2.1	0.0049±0.0022	UD	0.0010±0.0001
HPRT					
Initial log-phase	0.03±0.06	2.1±0.4	0.0089±0.0024	UD	0.0008±0.0001
Terminal log-phase	0.44±0.17	3.5±1.7	0.0050±0.0022	UD	0.0011±0.0001

	Cells				
sGC1α_2	WI-38	C-4 I	C-33 A	SiHa	ME-180
GAPDH					
Initial log-phase	UD	UD	0.14±0.07	7.6±2.7	1.1±0.11
Terminal log-phase	UD	UD	0.10±0.02	8.2±4.5	1.1±0.11
HPRT					
Initial log-phase	UD	UD	0.08±0.04	11.0±4.1	1.1±0.26
Terminal log-phase	UD	UD	0.10±0.02	8.7±4.3	1.2±0.13

UD, Gene expression undetectable.

Table III. *Expression of cyclic nucleotide phosphodiesterase 5 (PDE5A) was determined as described in the Material and Methods and the expression was calculated in relation to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT). The results are shown as the mean value±SD from three experiments.*

	Cells				
PDE5A	WI-38	C4-I	C-33A	SiHa	ME-180
GAPDH					
Initial log-phase	38.1±10.1	1.6±0.3	1.2±0.3	2.0±0.3	5.2±1.2
Terminal log-phase	57.9±5.2	2.6±1.1	0.5±0.2	1.6±0.2	6.8±0.7
HPRT					
Initial log-phase	71±25	1.1±0.2	0.64±0.17	3.0±0.5	5.4±1.3
Terminal log-phase	186±27	1.9±0.9	0.55±0.16	1.7±0.6	7.5±0.9

Table IV. *Expression of ATP-Binding Cassette transporter, member 5 of subfamily C (ABCC5) was determined as described in the Material and Methods and the expression was calculated in relation to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT). The results are shown as the mean value±SD from three experiments.*

	Cells				
ABCC5	WI-38	C-4 I	C-33 A	SiHa	ME-180
GAPDH					
Initial log-phase	0.41±0.16	2.4±0.6	0.06±0.23	1.0±0.1	2.0±0.4
Terminal log-phase	0.50±0.07	4.1±2.0	0.1±0.01	1.4±0.06	3.0±0.2
HPRT					
Initial log-phase	1.4±0.5	1.7±0.4	0.03±0.01	1.4±0.2	2.0±0.4
Terminal log-phase	2.3±0.4	3.0±1.6	0.1±0.01	1.5±0.6	3.3±0.3

Discussion

NO, synthesized by NOS, stimulates sGC, whereas the elimination of cGMP is taking place through metabolism by cnPDEs into GMP and cellular efflux by ABCC5. One or a combination of these biokinetic processes with increased biosynthesis, the reduced degradation or the increased cellular efflux is responsible for increased urinary cGMP levels observed in patients with cervical carcinoma (8, 9).

ABCC5, previously termed MRP5, plays an important role in the elimination of physiological intracellular cGMP (23). In some types of cancer, among them gynaecological malignancies, the increase of extracellular cGMP level has been reported as a sensitive, early and reliable marker for disease relapse. This was demonstrated after treatment of ovarian cancer (4-7) and cervical carcinoma (8, 9). A kinetic analysis of cGMP showed that an increased rate of appearance in plasma, *i.e.* cellular efflux, rather than an enhanced elimination explained the increase of urinary cGMP levels (10). Furthermore, in previous studies of the same cell lines as the ones used in the present work, changes in the ratio between extra- and intracellular cGMP levels suggested that cellular cGMP extrusion increased in parallel with cell growth (24, 25). This *in vitro* model can mimic the growing tumour bulk *in vivo*. More recently, altered expression of ABCC5 in relation to cancer prognosis and therapy resistance has been reported. In acute lymphoblastic leukaemia, high expression of *ABCC5* and some other *ABCC* genes was associated with reduced relapse-free survival in children and adults (26). This also supports the idea that cellular extrusion of cGMP reflects disease activity in patients with leukaemia (27). Cyclic GMP levels were normalized in all patients with complete remission and remained in the normal range during the remission period. In the patients who experienced disease relapse, an early increase in cGMP levels to the pre-treatment value was observed (27, 28). In gastrointestinal cancer, urinary cGMP levels were reportedly elevated during active disease (10, 29). More recent studies suggest an up-regulation of ABCC5 in cancer cells as

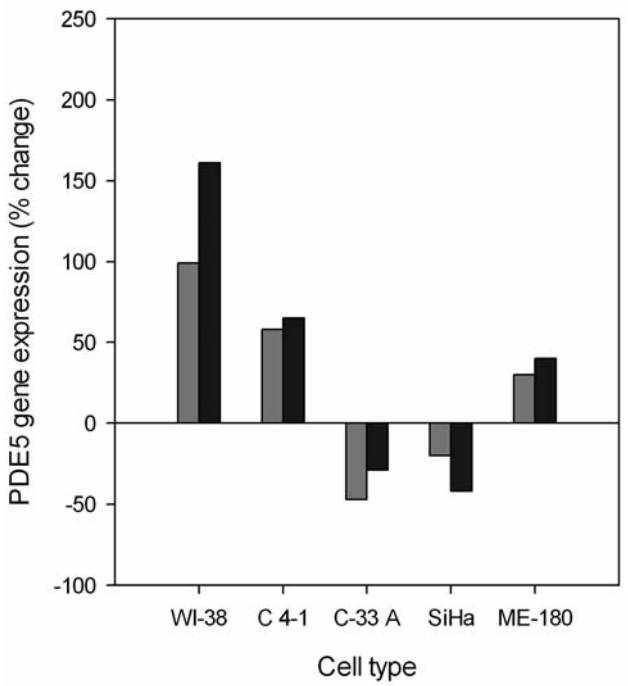

Figure 3. *Changes in expression of cyclic nucleotide phosphodiesterase 5 (PDE5) during exponential growth. PDE5 expression was calculated in relation to that of GAPDH (red) and HPRT (black). The results are presented as the mean value of three growth curves.*

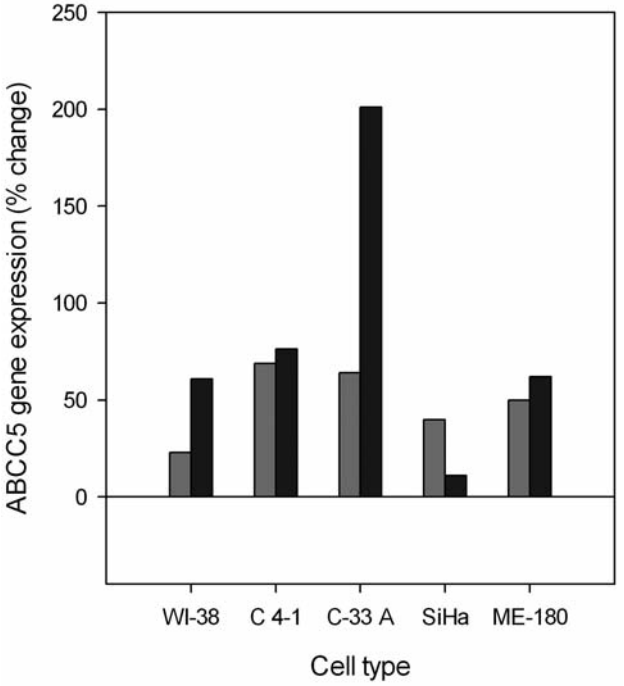

Figure 4. *Changes in expression of ATP-Binding Cassette transporter, member 5 of subfamily C (ABCC5) during exponential growth. ABCC5 expression was calculated in relation to that of GAPDH (red) and HPRT (black). The results are presented as the mean value of three growth curves.*

being responsible for the increased extrusion of cGMP. In pancreatic cancer, the *ABCC5* mRNA levels were shown to be significantly higher within the tumour compared to normal tissue (30) and, together with *ABCC3* and *ABCC4, ABCC5* they were up-regulated in 5-fluorouracil-resistant (5-FU) pancreatic carcinoma cells (31). Furthermore, in human oesophageal carcinomas, the most frequently amplified genomic region was chromosome 3q, including the *ABCC5* gene (32). In metastatic colorectal cancer, it was recently suggested that ABCC5-expressing circulating tumour cells may indicate resistance to drugs such as 5-FU (33).

In the present study ABCC5 expression increased in all transformed cell lines during the exponential growth phase. This model may mimic the growing tumour *in vivo* with increasing cGMP efflux per cancer cell. A similar change was observed for the non-transformed fibroblasts, in apparent contradiction to extracellular cGMP, as being a useful marker of malignant growth. However, in the fibroblasts, the effect was counteracted by an increased PDE5 expression (≈130%), by far exceeding the effect of increased ABCC5 expression (≈40%).

Long before the 11 families of cnPDEs were described (21), elevated cyclic nucleotide levels (34) in normal compared with neoplastic cells, were observed. In our former studies of intracellular cGMP levels with the same cell types as the one used in the present work, the levels tended to be lower in the cancer cells than in control cells. However, in both normal and transformed cells intracellular cGMP levels decreased during growth (24, 25). PDE5 has been purified and characterized as a cytosolic isoenzyme that specifically hydrolyzes cGMP and it is inhibited by zaprinast (35). Its overexpression in cancer is reported (36). Inhibition of PDE5 in cancer cell lines results in

Table V. *Summary of expression (Tables I-IV) and change (Figures 1-4) during the exponential growth period between the lag and plateau phase. The gene expression of inducible nitric oxide synthase (iNOS), soluble guanylyl cyclase (sGC) subunit α_1 and α_2, cyclic nucleotide phosphodiesterase 5 (PDE5A) and ATP-Binding Cassette transporter, member 5 of subfamily C (ABCC5) was related to the expression of the house-keeping genes GAPDH and HPRT. Undetectable (–), low (+), moderate (++) and high (+++) expression and increase (↑), decrease (↓), or no change (↔) in expression are shown.*

	Cells				
Gene	WI-38	C-4 I	C-33 A	SiHa	ME-180
iNOS	–	–	++ (↓)	++ (↓)	+ (↑)
sGCα_1	+ (↑↑)	++ (↑)	+ (↓)	–	+ (↑)
sGCα_2	–	–	+ (↔)	++ (↔)	++ (↔)
PDE5A	+++ (↑)	++ (↑)	+ (↓)	++ (↓)	++ (↑)
ABCC5	+ (↑)	++ (↑)	+ (↑)	++ (↑)	++ (↑)

mitotic arrest (37). The present study unveiled two features of the control cells, their high expression of PDE5 and its increased expression during growth (≈130%), distinguishing them from the transformed cells. In the malignant cells, the change in PDE5 expression ranged from –40% to +60%, and thus did not counteract the increase of ABCC5 expression.

Basal cGMP levels contribute to protection from various apoptotic stimuli (38), including protection of uterine epithelial and ovarian cells from spontaneous apoptosis (39, 40). Even if the precise mechanisms by which cGMP regulates cell fate are unknown, cGMP-dependent reduction in p53 may be involved (40). The present study showed that normal cells expressed the cGMP α_1 subunit at a low level which dramatically increased during growth, whereas the α_2 subunit was undetectable at both growth stages. Expression in the transformed cells was not consistent, as C-4 I cells expressed the α_1 but not α_2 subunit, whereas SiHa cells expressed the α_2 but not α_1 subunit. C-33 A and ME-180 cells expressed both subunits at low to moderate levels.

NO produced by NOS is an important stimulator of sGC. The NOS enzymes have been demonstrated to be present in human gynaecological cancer, among them cancer of the uterine cervix (12, 41). The present study showed that lung fibroblasts did not express iNOS, neither in the initial nor in the final stage of growth. The pattern of expression in transformed cells was also confusing with very low to low iNOS levels in C-4 I and ME-180 cells and with moderate levels in C-33 A and SiHa cells, which decreased during growth.

Table V summarizes the observations recorded in the present study. Assuming that the WI-38 control cells reflect the situation in normal tissues, it appears that during physiological proliferation, iNOS remains un-expressed. The expression of sGC α_1 subunit is low when the cells are at rest, but increases markedly during exponential growth; PDE5 is highly expressed and increases during growth; and ABCC5 exhibits low to moderate expression, but increases during exponential growth. The only gene that showed consistency in normal and in all malignant cells was *ABCC5*, with moderately high expression which increased during exponential growth. The increased transport capacity (ABCC5 V_{max} ↑) may account for the elevation of extracellular cGMP observed in some types of cancer since PDE5 expression exhibited minor changes. In contrast, the normal cells counteract this (ABCC5 V_{max} ↑) with a very high PDE5 expression; this reduces the substrate concentration (intracellular cGMP ↓) available for the ABCC5 transporter.

In conclusion, the present study unveils differences in the regulation of cGMP biokinetics between normal and malignant cells. Whether such differences can be employed in cancer diagnostics and therapeutics remains to be seen.

Acknowledgements

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