

Does the PGC-1/PPAR γ pathway play a role in Com-1/p8 mediated cell growth inhibition in prostate cancer?

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Abstract. Com-1, candidate of metastasis-1, also known as p8, has been shown to regulate the growth and apoptosis of cancer cells and is associated with the disease progression in human cancers including prostate cancer. In the current study, we investigated a potential mechanism underlying the anti-cancer action of Com-1/p8 in human prostate cancer. Human prostate cancer cells were used. Full-length Com-1 cDNA was isolated from normal mammary tissues. Ribozyme transgenes that specifically target human Com-1 were constructed using the pEF6/V5-His vector. Com-1 interacting proteins were determined using immunoprecipitation method. Cell growth and invasiveness were investigated using *in vitro* methods. Using immunoprecipitation and Western blotting, Com-1 was found to be cross-precipitated with PGC-1, a coactivator of peroxisome proliferator activated receptor (PPAR)- γ , but not PPAR- γ itself. Elimination of Com-1 from prostate cancer cells resulted in a reduced response of the cells to ciglitzone, a PPAR- γ agonist, whereas forced expression of Com-1 rendered cells more responsive to ciglitzone. We further demonstrated that the overexpression of Com-1/p8 resulted in changes in the expression of the PGC-1 responsive gene, fatty acid synthase (FAS). Com-1 may act as a tumour suppressor in human prostate cancer cells. The potential tumour suppressive effect of Com-1 is at least partly via its interaction with PGC-1, the PPAR- γ coactivator.

Introduction

Com-1, candidate of metastasis-1, otherwise known as p8, which was discovered from metastatic breast tumours of nude rats, has been under active investigation in recent years.

Although initially thought to play a role in promoting metastasis and to have some degree of mitogenic activity (1,2), subsequent investigations have shown that the molecule appears to play a more diverse role in cancer, depending on the cell function and tumour type. In thyroid cancer (3), in fibroblasts (4), and in pancreatic cancer (5,6), Com-1 appears to be associated with the aggressiveness of the tumour cells. However, in other tumour types, Com-1 has a different expression pattern. In breast cancer cells, Com-1 is associated with 1,25-Dihydroxyvitamin D3 induced growth inhibition which coincides with a rise of Com-1 (7). In clinical breast cancer, low levels of Com-1 transcript are associated with a poor clinical outcome of the patients (8). In pancreatic cancer, the overexpression of Com-1/p8 by transfection resulted in growth inhibition (9), and p8-silenced cells by way of an antisense approach grew more rapidly than control cells transfected with the empty retrovirus. It has also been shown that inhibitors to MEK1/2 and JNK can upregulate p8 expression, and that in fibroblasts, p8 is a cell growth inhibitor that facilitates apoptosis induced by DNA damage (10) which may involve p53.

The p8 polypeptide is a phosphoprotein subject to constitutive degradation by the ubiquitin/proteasome system mediated by phosphatidylinositol 3-kinase and protein kinase B/Akt. By contrast, stabilization of the p8 protein requires glycogen synthase kinase-3 (11). Garcia-Montero *et al* (12) have shown that TGF- β -1 can activate Com-1/p8 expression, which in turn enhances the Smas-transactivation in the TGF- β pathway. Com-1/p8 is primarily a nuclear protein and has been suggested to possibly be involved in the p27kip1 and p21cip1 pathway. We have recently shown that Com-1 interacts with ER- β and counteracts oestrogen-mediated effects on breast cancer cells (13).

Here we report the potential interaction between Com-1 and the peroxisome proliferator-activated receptor- γ coactivator-1 (PGC-1). The human transcriptional coactivator PPAR- γ coactivator-1 α (PGC-1 α), identified from a human cDNA library in a steroid-responsive yeast strain (14,15), has been characterized as a tissue-specific coactivator that enhances the activity of many nuclear receptors, coordinates transcriptional programs important for energy metabolism and

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acts as a broad regulator of cellular energy metabolism. The orphan nuclear receptor estrogen-related receptor α (ERR- α) was identified in a yeast two-hybrid screen of a cardiac cDNA library as a novel PGC-1 α -binding protein. It has been reported that PGC-1 α and ERR- α interact with each other and this interaction is distinct from that of other nuclear receptor PGC-1 α partners, including PPAR- α , hepatocyte nuclear factor-4 α and estrogen receptor α . Thus, ERR- α and ERR- γ are novel PGC-1 α interacting proteins. It would also appear that PGC-1 α selectively binds transcription factor partners in the regulation of energy metabolism (16). Few responsive genes are known to PGC-1, including Farnenoid X receptor (Fxr), nucleus receptors (NrF1) and fatty acid synthase (FAS) (17,18).

Following our recent study which revealed a reduction of Com-1 in prostate tumours and that Com-1 may act as a tumour suppressor using *in vitro* and *in vivo* models with prostate cancer (19), we examine the potential role of the PPAR- γ and PGC-1 pathway in the action of Com-1 in prostate cancer cells and report that in prostate cancer cells interaction between Com-1/p8 and PGC-1 represents a novel mechanism for the action of Com-1.

Materials and methods

Materials. Human prostate cancer cell lines PC-3, DU-145, CA-HPV-10 and LN-Cap were obtained from ATCC (American Type Cell Culture, Rockville, MD). RNA extraction and RT kits were obtained from AbGene Ltd. (Surrey, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA) and synthesised by Invitrogen Ltd. (Pasley, UK). Molecular biology grade agarose and DNA ladder were obtained from Invitrogen, and the master mix for routine PCR and quantitative PCR, AbGene. Monoclonal antibody to human PPAR- γ , polyclonal antibody to human PGC-1 and rabbit anti-human Com-1 (p8) polyclonal antibody were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat antibodies were from obtained from Sigma (Little Chalfont, UK) and a biotin universal staining kit, from Vector Laboratories (Nottingham, UK). Ciglitizone was purchased from Sigma Ltd.

Immunocytochemical staining of Com-1. Cancer cells were fixed with 4% formaldehyde and permeabilised with 0.1% Triton-X100. After blocking with 10% milk, primary antibodies (1:150 dilution from stock) were added to the cells for 1 h, followed by extensive washing. Respective HRP-conjugated secondary antibody was then added for 1 h, followed by extensive washing and DAB for colour development. Cells were then photographed using a digital camera, for evaluation of cytoplasmic and nuclear staining. The slides were then submerged overnight in xylene to remove the cover slips, followed by descending grades of ethanol washing. The slides were then counterstained with hematoxylin and remounted for photographing and presentation. Images from non-counterstained slides were processed using Optimas software (Optimas, Bothell, WA, USA). The staining intensity was determined using a method previously described (20), where between 50-80 cells of each setting were randomly analysed. Mean arbitrary intensity was calculated for the cytoplasmic and nuclear regions.

RNA extraction, cDNA synthesis and preparation of Com-1/p8 knockdown and human Com-1 overexpressing cells. RNA was extracted from cells and the concentration of RNA was determined using a UV spectrophotometer. Reverse transcription was performed using a RT kit with an anchored oligo-dt primer (AbGene), using 1 μ g of total RNA. The quality of the cDNA was verified using β -actin primers.

The hammerhead ribozyme transgenes and Com-1 expression cassette were prepared as in previous studies (13,19,21). The following stably transfected cells from PC-3, DU145 and CA-HPV-10; Com-1 knockout cells, PC3^{Δcom1}, DU145^{Δcom1} and CA-HPV^{Δcom1}; Com-1 overexpressing cells, PC-3^{com1Exp}, DU145^{com1Exp}, and CA-HPV^{com1Exp}; and plasmid only control cells, PC3^{pEFa}, DU145^{pEFa} and CA-HPV^{pEFa}, were used together with the respective wild-types.

Immunoprecipitation and Western blotting. Cells were extracted using a lysis buffer, containing 2.4 mg/ml Tris, 4.4 mg/ml NaCl, 5 mg/ml sodium deoxycholate, 20 μ g/ml sodium azide, 1.5% Triton, 100 μ g/ml PMSF, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin, for 30 min. Protein concentrations were measured using fluorescamine and

Table I. PCR primers.

	Sense (5'-3')	Antisense (5'-3')
Com-1 quantitation and screening	cctggatgaatctgacctc	actgaacctgaccgtacacaagcagcttctcttgggtg
Com-1 expression	atggccaccttcccac	actgcgcctgcccctcg
Com-1 ribozyme-8	ctgcagcttctcttgggtgcctgatgagtcctgagga	actagtggaggccggaagggttctgctcctcaggact
Com-1 ribozyme-9	ctgcagctgggtgctggtgctgatgagtcctgagga	actagtatggccaccttttctgctcctcaggact
Ribozyme screening	ctgatgagtcctgaggacgaa	ttcgtcctcaggactcatgac,
PGC-1	agtacaacaatgagccttcaa	catcaaatgagggaatc
PPAR- γ	ctatggagttcatgctgtg	gtactgacatttattt
Fxr	gggaactgaaaatgactcaa	actgaacctgaccgtacatcctgaagcttcttactgc
NrF1	atggcctctttatggcagat	actgaacctgaccgtacacacagctgtgtacatgct
FAS	tgccgcctataggacag	actgaacctgaccgtacagtgagctgtccagggtga
β -actin	gctgatttgatggagtggaa	tcagctactgttcttgagtga

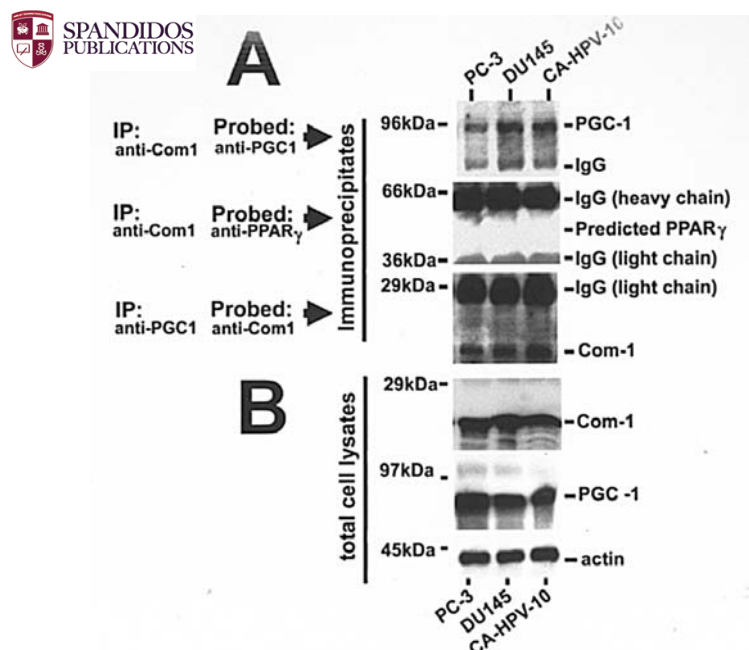


Figure 1. Interaction between Com-1 and PGC-1. (A) Cross immunoprecipitation between Com-1 and PGC-1. Cell lysates were either precipitated with anti-Com-1 (top two panels) or with anti-PGC-1 antibody. The precipitates were separated on either SDS-PAGE (top two panels) or Tricine-PAGE (bottom panel) and probed with the respective antibodies. Com-1 and PGC-1 cross-precipitated with each other, however, no interaction was seen between PPAR- γ and Com-1. (B) Expression of all three proteins in prostate cancer cell lines.

quantified by using a multi-fluoroscanner (Denly, Sussex, UK). The samples were then boiled at 100°C for 5 min before clarification at 13,000 g for 10 min. Equal amounts of protein from each cell sample (controls and treated) were added to an 8% poly-acrylamide gel. For immunoprecipitation, prostate cancer cells were pelleted and lysed in the same buffer with 1 mM sodium orthovanadate. Anti-Com-1/p8 (80 μ g) or anti-PGC-1 antibody was added to equal amounts of protein for 1 h with constant agitation, followed by the addition of protein A/G agarose for an additional 1 h and washing with the same lysis buffer. The immunoprecipitate was solubilised with a sample buffer that contained 5% 2-mercaptoethanol. The samples were then separated on 10% SDS-PAGE or 12.5% Tris-Tricine PAGE (for Com-1 only). Protein blots were

probed with a panel of antibodies (n=64) from a pool of antibodies in our laboratories, followed by washing and subsequent probing with appropriate peroxidase-conjugated secondary antibodies. Protein bands were visualised with a chemiluminescence (ECL) kit.

In vitro cell growth assay. This was modified from a previously described method (22). Cells were plated into a 96-well plate at 2,500 cells/well, with or without treatment with ciglitizone (0.1-100 μ M final concentration). Cells were fixed in 10% formaldehyde at the day of plating and daily for 6 subsequent days after plating, and then stained with 0.5% (w/v) crystal violet. Following washing, the stained crystal was extracted with 10% (v/v) acetic acid and the absorbance was determined using a multiplate reader. The growth of cells are shown in this study as percentage of the respective untreated cells at a given day.

Results

Interaction of Com-1 with PGC-1, a PPAR- γ coactivator. To identify the potential binding partners of Com-1, we first used immunoprecipitated proteins that interacted with Com-1, using the anti-Com-1 antibody for immunoprecipitation. The precipitates were subsequently separated on multiple gels and blots probed with a panel of 64 antibodies that recognise potential nuclear as well as other proteins. From the panel, PGC-1, but not PPAR- γ , was one of the few proteins that co-precipitated with Com-1 (Fig. 1). Using reverse precipitation, precipitation with anti-PGC-1 antibody and probing with anti-Com-1 antibody, the interaction between the two proteins was verified (Fig. 1).

Modification of Com-1 expression results in differential growth response to PPAR- γ . To test if Com-1 is involved in PPAR- γ /PGC-1 mediated cell growth inhibition in response to the PPAR- γ agonist, we first generated a panel of genetically modified (GM) prostate cancer cell lines, whose expression of Com-1 was either knocked down by ribozymes or increased by forced expression (Fig. 2) (19).

The effects of ciglitizone on the growth of these cells were evaluated. Ciglitizone inhibited cell growth over a range of concentrations (Fig. 3, open circles). However, Com-1 knockout prostate cancer cells had reduced sensitivity,

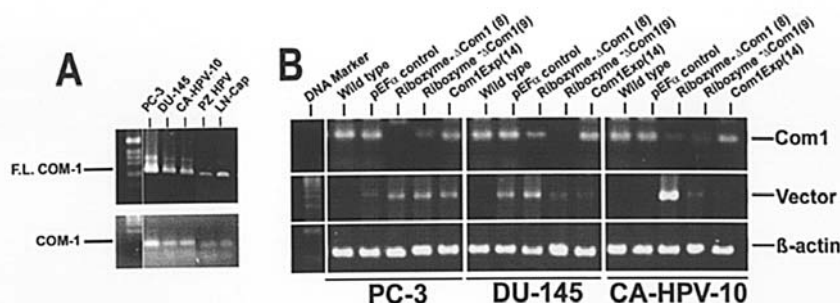


Figure 2. Expression of Com-1 in prostate cancer cells (A) and genetic manipulation (B). All five prostate cancer cell lines expressed Com-1 mRNA (F.L. Com-1, full-length Com-1; bottom panel in A, amplified internal region of Com-1). Two ribozyme transgenes had knockdown expression of Com-1 mRNA from all three prostate cancer cells (B). (adapted from ref. 19.)

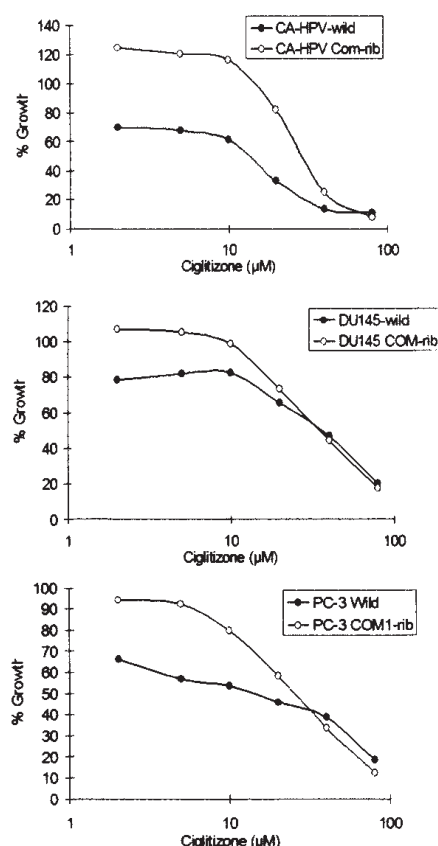


Figure 3. Loss of Com-1 expression and change of cell response to ciglitizone. Shown are representative responses from three prostate cancer cells with which their expression of Com-1 was knocked down by Com-1 ribozyme transgene (Com1-rib). Shown are percentages of cell growth over a 5-day period. Top, CA-HPV cells; middle, DU145 cells; bottom, PC-3 cells.

although not completely, to the growth inhibition of ciglitizone (Fig. 3, solid circles). Fig. 3 shows the cell response on day 5. Data for all the respective cells including wild-type, control plasmid-transfected, ribozyme-transgene transfected and expression vector-transfected cells are summarised in Table II.

We further treated the aforementioned GM cells with ciglitizone, an agonist to PPAR- γ . As shown in Fig. 4 (top panel), ciglitizone increased the Com-1 protein levels in both wild-type PC-3 and PC3^{pEFa} as shown in Western blotting. However, the agonist had no marked effect on PGC-1 expression.

We further analysed the cellular and nuclear staining of Com-1, PGC-1 and PPAR- γ using immunocytochemistry (ICC) and Western blotting (WB). As shown in Fig. 4, treatment of wild-type PC-3 cells seems to only marginally increase the amount of Com-1 (Fig. 4: top, WB; bottom panel, ICC). This marginal increase appears to be in the nuclear fraction.

Modification of Com-1 expression and results in changes of PGC-1 responsive genes. To assess if the manipulation of Com-1 has an impact on the responsive genes of PGC-1, we evaluated the expression of FAS, FxR and NrF1, which are known PGC-1 responsive genes. We evaluated these gene products in Com-1 modified cells, PC3^{Δcom1}, DU145^{Δcom1}, CA-HPV^{Δcom1}, Com-1 overexpressing cells, PC-3^{com1Exp}, DU145^{com1Exp}, and CA-HPV^{com1Exp}, plasmid only control cells, PC3^{pEFa}, DU145^{pEFa} and CA-HPV^{pEFa}, used together with the respective wild-types. The overexpression of Com-1 rendered cells to express lower levels of FAS, but not FxR and NrF1, compared with wild-type and transfection control cells (Fig. 5).

Discussion

Com-1 interacts with PGC-1, but not PPAR- γ . We conducted a co-precipitation test using a panel of antibodies, and found that the peroxisome proliferator-activated receptor- γ (PPAR γ) coactivator-1, PGC-1, is one of the few proteins that co-precipitated with Com-1. The human transcriptional coactivator PPAR- γ coactivator-1 α (PGC-1 α), identified from a human cDNA library in a steroid-responsive yeast strain (16,17), has been characterized as a tissue-specific coactivator that enhances the activity of many nuclear

Table II. Com-1 expression and ciglitizone-regulated cell growth (72 h).

Cell line	Variant	Growth (%)		
		1 μ M	5 μ M	20 μ M
PC-3	Wild-type	93.3	93.3	56.4
	pEFa control	91.7	89.9	60.2
	Com1Rib	138.2	101.6	63.9
	Com1Exp	108.8	80.7	40.0
DU-145	Wild-type	92.0	96.9	85.9
	pEFa control	94.1	98.4	87.2
	Com1Rib	107.0	97.9	90.4
	Com1Exp	92.7	88.6	41.9
CA-HPV	Wild-type	104.2	105.7	72.79
	pEFa control	108.3	101.7	93.3
	Com1Rib	102.8	100.6	96.6
	Com1Exp	98.1	70.2	37.6

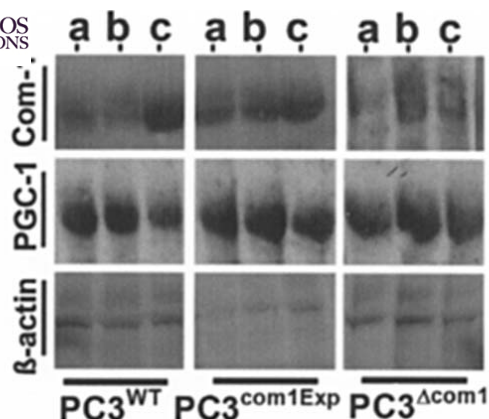


Figure 4. Effects of ciglitizone on protein expression of Com-1 and PGC-1 in PC-3 cells. Top: cells were treated with (a) medium, (b) medium with 1% DMSO and (c) ciglitizone (5 μ M). Increased levels of Com-1 were seen with wild-type and transfection control cells, but not in Com-1 knockdown cells (top panel). Changes in PGC-1 in these cells were not significant (middle panel). β -actin was used as a housekeeping control. Bottom, immunocytochemical staining of Com-1 in wild-type PC-3 cells following treatment with medium (left panel) or with ciglitizone (5 μ M, middle panel). The changes in the staining intensity of the cells are given in the right panel.

receptors, coordinates transcriptional programs important for energy metabolism and is a broad regulator of cellular energy metabolism. PGC-1 promotes transcription through the assembly of a complex that includes the histone acetyltransferases steroid receptor coactivator-1 (SRC-1) and CREB binding protein (CBP)/p300 (23). PGC-1 acts to coordinate the process of metabolic adaptation in the liver, by interacting with HNF4 α and cAMP-response element-binding protein (CREB) (24,25). PGC-1 is a coactivator of MEF2C, a muscle-selective transcription factor, and can control the level of endogenous GLUT4 gene expression in muscle (26). Few PGC-1 responsive genes are known. Farnecoid X receptor (Fxr), nuclear receptor family group H members and fatty acid synthase are amongst the known genes (17,18). The over-expression of PGC-1 can decrease the expression of fatty acid synthase. The current study has shown that the overexpression of Com-1 in prostate cancer cells also results in the reduction of fatty acid synthase expression. However, manipulating Com-1 expression has not affected the

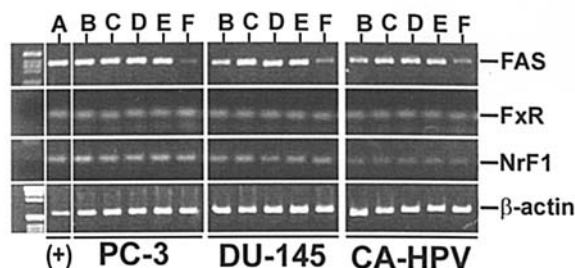


Figure 5. Expression of Com-1 and the impact on the expression of putative PGC-1 responsive genes FAS (fatty acid synthase), Fxr, and NrF1. (A) Positive control from normal human tissues, (B) wild-type cells, (C) control transfected cells (with pEFa plasmid), (D) Com-1 ribozyme transfected cell-1, (E) Com-1 ribozyme transfected cell-2, and (F) cells with Com-1 forced expression.

expression of NrF1 and Fxr. Taken together, Com-1 may operate with PGC-1 in regulating PGC-1 responsive genes, including fatty acid synthase.

The impact of Com-1 and PGC-1 requires further investigation. However, PGC-1 is involved in the transcriptional regulation of the UCP1 gene in humans and mediates the effects of PPAR- α and PPAR- γ agonists and retinoic acid (27) and is a coactivator for RXR α (28). PGC-1 has also been implicated in the cachectic response and oxidative metabolism in skeletal muscle (28-31). These collectively suggest an important role for Com-1 in the metabolic response in malignancies.

A potential role for Com-1 in the PGC-1/ER connection.

PGC-1 is a bona fide ER α coactivator, which may serve as a convergence point between PPAR- γ and ER α signalling (32). A PGC-1 related coactivator, PERC (PGC-1 related estrogen receptor coactivator), is known to interact with the AF2 domain of ER α in an estrogen-dependent manner. PERC and PGC-1 show distinct preferences for enhancing ER α in different promoter contexts. PERC enhances the ER α -mediated response to the partial agonist tamoxifen, while PGC-1 modestly represses it. The two coactivators are likely to mediate distinct, tissue-specific responses to estrogens (33). Akt can mediate the downregulation of peroxisome proliferator-activated receptor (PPAR)- γ coactivator-1 (PGC-1) and PPAR- α (34). Recently, we reported that Com-1 forms a complex with ER- β in human breast cancer cells (8,13). In breast cancer cells, the loss of Com-1 resulted in cells responding more vigorously to oestrogen with a rapid increase in cell growth. Vice versa, the overexpression of Com-1 in breast cancer cells resulted in the cells being less responsive to oestrogen and less invasive. We suggested that Com-1, by forming a complex with ER- β and PGC-1, acts as a tumour suppressor in human solid tumours including prostate and breast cancers (8).

The role of Com-1 in prostate cancer cells.

We and others have reported that PGC-1 expression, together with PPAR- γ , is reduced in human cancer (31,35). Furthermore, ER- β , which plays an opposite role to ER- α in cancer cells, has also been reported to be reduced in certain types of cancer such as breast cancer.

Peroxisome proliferator-activated receptor- γ (PPAR- γ) belongs to a nuclear hormone receptor super-family that regulates gene expression. PPAR- γ contains DNA, lipid and A/B binding domains that recognise DNA response elements in the promoters of their target genes and interact with lipid ligands and prostaglandins (PGJ2). Chang *et al* has shown that ligands of PPAR- γ such as troglitazone, BRL49653, 15S-hydroxyeicosatetraenoic acid and 15-deoxydelta12,14-prostaglandin J2 are capable of inducing apoptosis of cancer cells and inhibiting tumourigenesis (36,37). It has also been demonstrated that PPAR- γ forms a complex with its coactivators (PGC-1 and -2) and in association with other transcription factors such as SRC-1 and CREB binding proteins (38-40) regulates the expression of a number of target genes including the cMet protooncogene, E-cadherin and the cyclin regulator p27^{Kip1}. Studies by Hsistake (41) demonstrated that PPAR- γ agonists are capable of reducing the growth and progressive activity of prostate cancer cells as well as inhibiting the transcription of genes such as cMet and HGF both of which are associated with tumour progression.

Anomalies in PPAR group members have been shown to cause tumourigenesis in both human cancers and animal models. Previous studies have shown (42) that poly-unsaturated fatty acids activate PPAR- γ levels in cancer cells. PGC-1, as a key modulator of hepatic gluconeogenesis and as a central target of the insulin-cAMP axis in liver, regulates mitochondrial biogenesis and function (15,43). The transcriptional activity of PPAR γ may not only be decreased by mutation but also by downregulation of the coactivator PGC-1 of PPAR- γ (44). Recently, we have also reported, for the first time, that the mRNA level of PGC-1 is also reduced in human breast cancer and the reduction is associated with an aggressive nature of the breast tumours (35). Taken together, it is suggested that in human tumours including prostate cancer, the loss and/or reduction of PGC-1 and Com-1 in cancer cells, particularly in the nuclear fraction, may act both on the control of transcription of tumour suppressor genes and cell growth. This would allow cancer cells to gain rapid growth and aggressiveness. A few other theories have been recently reported with regards to the mechanism of Com-1-regulated cell growth and death. Com-1/p8 has been shown to be one of the key modulators in the antitumour (pro-apoptotic) effects of the cannabinoids, and p8 mediates its apoptotic effect via upregulation of the endoplasmic reticulum stress-related genes ATF-4, CHOP, and TRB3 (45). Furthermore, the interaction between Com-1/p8 and prothymosin α (ProT α) has been reported to be key to staurosporine-induced apoptotic response in HeLa cells (6). The interplay between Com-1, PGC-1 and ProT α warrants further investigation.

In conclusion, the current study has shown that Com-1/p8 is a potential tumour suppressor in human prostate cancer. One of the potential mechanisms underlying the action of Com-1 is its interaction with PGC-1. Levels of Com-1 influence cellular responses to PPAR- γ agonists. Com-1 may have important therapeutic implications as well as prognostic implications.

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