TRANSCRIPTIONAL UPREGULATION OF GASTRIN IN RESPONSE TO PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA AGONIST TRIGGERS CELL SURVIVAL PATHWAYS

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Peroxisome proliferator-activated receptor γ (PPARγ) are members of the largest nuclear hormone receptor family of transcription factors (1). PPAR gamma (PPARγ) plays an important role in adipogenesis, control of sensitivity to insulin, inflammation and atherosclerosis but recent studies also suggest that PPARγ is involved in cell cycle withdrawal. PPARγ can promote cell differentiation, exert an antiproliferative action and inhibit angiogenesis (2, 3). However, there are studies showing that activation of PPARγ promotes the development of colon cancer (4). These data are in sharp contrast with studies that attribute anticancer effects to PPARγ in gastrointestinal malignancies. Probably, the action of PPARγ on cell cycle and proliferation depends on the cell type and presence of other stimuli that predispose cells to cancer development. Amidated and non-amidated gastrins may play an important role in the proliferation and carcinogenesis of GI cancers. It is known that gastrin peptides activate phosphorylation of Protein Kinase B (PKB/Akt) and anti-apoptotic signalling but there is little known about the link between gastrins and PPARγ receptors in relation to apoptosis.

Key words: gastrin, pancreatic and colon cancer, Peroxisome proliferator-activated receptor γ, apoptosis

INTRODUCTION

The hormone gastrin amide is secreted by the G cells of the stomach, and is responsible for stimulation of gastric acid secretion. The initial translation product of the gastrin gene is the precursor molecule, preprogastrin (101 amino
acids), which is converted to progastrin (80 amino acids) by cleavage of N-terminal signal peptide. Progastrin is processed further within secretory vesicles by endo- and carboxy-peptidases to yield glycine-extended gastrins (35 and 18 amino acids, Gly-G). Progastrin and Gly-G represent the nonamidated forms of gastrin (NAGs). The C-terminus of glycine-extended gastrin34 is then amidated and, after further proteolytic cleavage, mature amidated gastrin17 (Gamide) is stored in G cells granules. Normally, progastrin and the glycine-extended gastrins comprise less than 10% of circulating gastrins (1).

Progastrin and its glycine-extended derivatives have previously been regarded as physiologically inactive, because C-terminal amidation was thought to be essential for the bioactivity via binding to the CCK-2 receptor. However, data have shown that gastrin precursors such as Gly-G stimulate proliferation in several cancer cell lines including pancreatic carcinoma cells and colon carcinoma cells (2). It has been also discovered that nonamidated gastrins exert potent growth factor effects in vitro on rat intestinal cells and 3T3 fibroblasts (3). Gly-G also accelerate the development of colorectal cancers in transgenic mouse models (4), modulate migration and apoptotic pathways in gastrointestinal cell lines (5, 6) and are elevated in patients with colorectal cancer (7). On the other hand gastrin is not mutagenic but behaves as a mitogenic factor. This hormone probably does not cause malignancy to arise, but it does stimulate the growth of pre-existing premalignant and malignant lesions (8). Gastrin is an important trophic factor for gastric epithelium and stimulates proliferation of the ECL cells of the stomach and proximal small intestine (8) and migration of gastric parietal cells (10).

In vivo growth effects of gastrins have been demonstrated in a number of mice models. Homozygous gastrin-deficient mice have histologically normal colonic mucosa but reduced proliferative index as measured by BrdU labelling (11). Infusion of Gamide into gastrin-deficient mice had no effect on the colonic proliferative index, but infusion of Gly-G increased the proliferative index by 80%. The results of these studies are consistent with the hypothesis that colorectal cancers produce progastrin but are deficient in the ability to process progastrin to Gamide (7).

There are also studies showing plenty of other stimuli that may modulate the important role that gastrin plays in gastrointestinal tract like melatonin, ghrelin, prostaglandins, nitric oxide and sensory nerves (12 - 14).

Amidated gastrins bind to CCK-1 and CCK-2 receptors. Both receptors belong to the family of seven transmembrane domain receptors (11). In addition both amidated and non-amidated gastrins bind to a low affinity binding site described as CCK-C receptor which is present in variety of tissues, including cancer cell lines (16). There are observations that CCK-1 or CCK-2 receptor antagonist did not block the proliferative effect of Gly-G confirming the involvement of a novel receptor (17), but no high-affinity Gly-G receptor has yet been cloned.

PPARs are members of the largest nuclear hormone receptor family of transcription factors (18, 19). The best characterised is PPARγ, which with the retinoid X receptor (RXR), affects cell proliferation and differentiation via target
gene transcription. The highest levels of PPARγ mRNA and protein are expressed in adipose tissue, large intestine and haematopoietic cells with intermediate levels in the kidney, liver, small intestine and other parts of gastrointestinal tract (19). PPARγ is activated by a number of fatty acid derivatives, insulin sensitizers (20, 21) and glucocorticoids which upregulate PPARγ expression in adipose tissue (21). On the other hand tumour necrosis factor α (TNFα) was reported to decrease PPARγ mRNA expression (22). However, little is known about how the PPARγ protein is regulated. A recent study suggests that PPARγ is activated by either naturally occurring compounds such as prostaglandin J₂ or synthetic molecules such as thiazolidinediones (19). Upon binding ligands a conformational change of PPARγ occurs and interaction between PPARγ and RXR is stabilised. This process allows the recruitment of many cofactors and enhances the transcription of target genes. Although many of the published studies imply an anti-proliferative effect of PPARγ agonists (23, 24), some authors have shown an opposite effect with PPARγ ligands promoting the tumour cells growth (25, 26). The effect of PPARγ is also controversial in literature and PPARγ ligands have been regarded as either inhibiting or stimulating angiogenesis (27, 28). That is why the link between gastrin precursors and PPARγ needs further investigation.

Therefore, the aims of the present study were to investigate the interactions between PPARγ and gastrin on carcinogenesis in terms of: (a) PPARγ and gastrin gene expression, and (b) circumvention of apoptosis using human pancreatic and colon cancer cell lines as in vitro models.

MATERIALS AND METHODS

Cell lines

PANC 1 (human pancreatic adenocarcinoma epithelial cell line, obtained from European Collection of Animal Cell Cultures no. 87092802) and HT29 (human epithelial colorectal adenocarcinoma, obtained from American Type Culture Collection no HTB-38). Cell lines were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 culture (Life Technologies, Inc., Paisley, United Kingdom) containing 10% heat-inactivated fetal bovine serum (FBS; Sigma, Poole, United Kingdom) at 37°C in 5% CO₂ and humidified conditions.

Tetrazolium-based growth assay (MTT)

Cells were plated out in 100µl aliquots into the middle 60 wells of a 96-well tissue culture plate (1x10⁴ cells/well). After overnight incubation at 37°C, 5% CO₂ dilutions of ciglitazone (source?) were added in 5 replicates per drug concentration (1-20µM); a control peptide (describe what this was) was used at the same concentrations. After 72 h incubation 50µl of methyl thiazolyl tetrazolium (MTT) solution was added to each of the test wells, and the plate was incubated for 4 h. Medium was removed and incorporated MTT dissolved in 75µl DMSO per well. The optical density at 550nM was measured.
**Transfection with siRNA**

Cell lines were transfected with a gastrin or PPARγ siRNA (Gastrin: AAGAAGAAGCCUAUGGAUGGA; PPARγ: AAGCCCUUCAUCUACUGUUGAC; Eurogentec, Romsey, United Kingdom) using siPORT Amine transfection reagent (Ambion, Huntingdon, United Kingdom) as previously described (18). A non-targeting siRNA (Eurogentec) was used as a control.

**RNA extraction and reverse transcription**

Total RNA was extracted from cell samples using RNA-Bee (Biogenesis; Poole, Dorset, United Kingdom). Reverse transcription was carried out using Superscript II reverse transcriptase reagents (InVitrogen, United Kingdom).

**Real-Time PCR**

Relative gene expression was determined by Real-Time PCR, using SYBR green dye. Optimized primers designed to amplify the gastrin gene were used in conjunction with reagents from the qPCR Core Kit SYBR Green I (Eurogentec, Romsey, United Kingdom). PCR assays were carried out on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Results were expressed relative to the housekeeping gene, Hypoxanthinephosphoribosyltransferase (HPRT), and control samples (either untreated with ciglitazone or transfected with siRNA) using the $2^{-\Delta\Delta CT}$ method.

**Western Blot Analysis**

The effect of ciglitazone (Sigma Aldrich, Dorset, United Kingdom) stimulation on Bcl-2 protein level and PKB/Akt phosphorylation was assessed by Western blotting using specific anti-Bcl-2 and anti-PKB/Akt, anti-Akt and anti-Actin antibodies (Cell Signalling Technologies, Beverly, MA). Biotin labelled swine anti-rabbit secondary antibodies (Dako Cytomation, Ely, United Kingdom) were used at 1/1000 followed by StreptABC complex/HRP (Dako Cytomation, Ely, United Kingdom). Chemiluminescent visualisation and protein detection were carried out using the Amersham enhanced chemiluminescence kit (Amersham, Little Chalfont, United Kingdom), with relative levels compared to untreated controls. Relative protein quantification was carried out using the Chemi Genius2 Biolmaging System (Syngene, Cambridge, UK) in conjunction with the GeneTools image analysis software (Syngene).

**Statistics**

For evaluation of in vitro data non-parametric Mann-Whitney U test was used.

**RESULTS**

**Exploration of the effect of ciglitazone on cell survival**

Growth effects of ciglitazone, a PPARγ agonist, and a control peptide were examined in the PANC1 and HT 29 cell lines using a tetrazolium based assay, 48 h after treatment. Ciglitazone exogenous administration caused a significant increase in growth of the PANC1 cell line at concentration of 10 - 20 μM but in the HT 29 cell line there was a significant decrease in cells growth after 48 h ciglitazone
treatment at concentration of 10 - 20 µM (p<0.01, non parametric Mann-Whitney U test, significant indicated by *) (Fig. 1). These data suggest that growth effects exerted by Ciglitazone via PPARγ probably depend on cancer cells type.

Examination the link between gastrin and PPARγ in terms of apoptosis

The potential role of gastrin in the anti-apoptotic effect was evaluated in PANC1 cells. Serum-starved PANC1 cells treated with gastrin showed increase either in PKB/Akt phosphorylation status or Bcl-2 and PPARγ protein reaching maximal levels at 60 min (Fig. 2). These findings suggest that the anti-apoptotic effects of gastrin are mediated, at least in part, through up regulation of PPARγ gene expression.

Effect of ciglitazone and PPARγ siRNA on gastrin gene expression

The effect of ciglitazone and PPARγ siRNA on gastrin gene expression was also evaluated. Treatment with 10µM ciglitazone alone resulted in a 2 - 3 fold
increase in (p<0.001) gastrin gene expression in comparison to the untreated control (Fig. 3). These findings suggest that in pancreatic cancer cell lines ciglitazone exerts its effects on gastrin gene via PPARγ.

DISCUSSION

Cancer progression depends on the activation of signal transduction pathways that control cell growth and survival. Recent studies showed that PPARγ is associated with antiproliferative effects (12 - 14) but there is also a growing number of reports indicating PPARγ promoting role in large intestine carcinogenesis (9). Gastrin, plays an important role in GI tract malignancies development and progression as an anti-apoptotic agent (1, 2, 3) but the relationship between PPARγ and gastrin is not yet established.

The main observation of our study is that the anti-apoptotic effects of PPARγ may be partially mediated via gastrin as transfection of PANC1 and HT29 cells with PPARγ siRNA resulted in down-regulation of gastrin gene expression in comparison to the untreated control and ciglitazone administration failed to
reverse that effect in the pancreatic cell lines. However, in the HT29 cells after PPARγ siRNA transfection there was an increase in gastrin gene expression after following 24 h ciglitazone administration compared to cells without the PPARγ agonist treatment. Moreover, transfection of the HT29 cells with gastrin siRNA prevented the increase in both PKB/Akt phosphorylation and expression of Bcl-2 protein in response to ciglitazone (data not shown). These data suggest existing of the feedback loop between gastrin and PPARγ in GI cancer cells as anti-apoptotic role of gastrin seems to be linked to PPARγ as PPARγ down-regulation resulted in significant decrease of gastrin gene expression in GI cancer cell lines. However, our data also showed that relationship between gastrin and PPARγ is slightly different in pancreatic and colon cancer cells probably due to dissimilar cells construction.

Therefore, we support hypothesis (19) that the action of PPARγ on the cell cycle and proliferation may depend on the cell type and presence of other stimuli that predispose cells to cancer development. More interestingly, different tumour cell may have different anti-apoptotic response to PPARγ agonists.
Moreover, to clarify the controversy in literature concerning the role of PPARγ in GI carcinogenesis it is important to take into consideration posttranslational modification of PPARγ which can be inactivated by mitogen-activated protein kinase kinase (MAPKK) by phosphorylation at Ser-82 (24). Many cancers have activated Ras which activation leads to an increase in MAPKK signalling. It may explain the reduced function of the PPARγ protein in the PAN1 cell line. There are recent studies showing that gastrin itself may decrease anti-cancer PPARγ effects by decreasing PPARγ activity via proteasomal degradation. Gastrin-promoted phosphorylation at Ser may lead to down-regulation of PPARγ activity. This may be an explanation for different observations concerning the effects of PPARγ and its agonists in GI malignancies.

In conclusion, in the present study we have shown that there is a strong correlation between PPARγ and gastrin in circumvention to apoptosis in pancreatic cancer cells as ciglitazone, increases anti-apoptotic potential through up regulation of the gastrin gene.

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REFERENCES


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