MOLECULAR MECHANISM OF PROTECTION AGAINST CHEMICALLY AND \( \gamma \)-RADIATION INDUCED APOPTOSIS IN HUMAN COLON CANCER CELLS

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The involvement of peroxisome proliferator-activated receptors (PPARs) in the cancer cell elimination through apoptosis is a generally accepted fact. However, some reports indicate that the activation of PPAR\( \gamma \) is directly responsible for carcinogenesis. Caco-2 cells, a human adenocarcinoma cells, were used as a model of colon cancer. Cell cultures (5x10^6 cell per dish) were pretreated for 24h with PPAR \( \gamma \) agonists ciglitazone (CI, 1x10^-6 M) and retinoic acid (RA, 1x10^-6 M) and part of the cultures were subsequently subjected to \( \gamma \)-radiation (photons) with therapeutic dose of 2.5 Gy. Total cellular RNA and proteins (cytoplasmic and nuclear) were isolated 24h after cultures irradiation or 48h after stimulation in the non irradiated part of experiment to preserve the equal growth time for all samples. \( \gamma \)-Irradiation of the cells abolished nuclear translocation of PPAR\( \gamma \) under its agonists treatment and preserved PPAR\( \gamma \) in the cytoplasmic pool. But it did not affect the HSP 70 expression in response to ciglitazone and retinoic acid. Moreover, combined \( \gamma \)-irradiation and CI/RA treatment of the cells changed the equilibrium between Bax and Bcl-2 mRNA to anti apoptotic state with increased expression of Bcl-2 and almost abolished expression of Bax. In conclusion, this paper provides an evidence for the anti-apoptotic action of PPAR\( \gamma \) agonists used along with the \( \gamma \)-radiation. Moreover, it shows that the up-regulated HSP70, in response to PPAR\( \gamma \) agonists in \( \gamma \)-irradiated cultures promotes cell survival.

Key words: PPAR\( \gamma \), ciglitazone HSP70, HSF-1, apoptosis, \( \gamma \)-radiation cancer

INTRODUCTION

Apoptosis is indispensable for embryogenesis, tissue homeostasis and the maturation of the immune system. Malfunctions of apoptosis have been apparent
with numerous human diseases, particularly the carcinogenesis and neurodegenerative disorders (1, 2, 3). The apoptotic cell death can be induced by a variety of different signals, including the activation of Fas or the receptors of tumor necrosis factor-alpha (TNF-α), growth factor deprivation, excessive DNA damage, treatment with chemotherapeutic drugs or stress such as heat shock or UV irradiation (4, 5, 6, 7).

The primary goal of the different strategies for neoplasm treatment is the induction of apoptosis in cancer cells. Chemotherapy and irradiation act on cancer cells by inducing apoptosis. The over expression of anti-apoptotic proteins and the subsequent interruption of the cell death pathway might explain why cancer cells are resistant to apoptosis induced by the anticancer therapy. It is well known that the high level of heat shock proteins (HSPs) in cancer cells is associated with metastasis, the poor prognosis and the resistance to radio as well as chemotherapy (8, 9).

It has been shown that the activation of PPARs, especially PPARγ leads to apoptosis associated with down-regulation of Bcl-2 expression (10). PPARs in the epithelial cells may be responsible for triggering of the caspase-3 dependent apoptotic pathways, which have been apparent in the other types of cells (11). PPARs are the transcription factors belonging to the steroid/thyroid/retinoid hormone nuclear receptors gene family (12, 13), which exhibits the distinct tissue distribution patterns and metabolic functions. They function as the ligand-dependent transcription factors that, upon heterodimerization with the 9-cis-retinoic acid receptor RXR, bind with specific response elements termed as the peroxisome proliferator-response element (PPRE), and thus regulate the expression of target genes (14).

Even though, the beneficial effect of agonists activated PPARs on the cancer cell elimination through apoptosis is a generally accepted fact (15, 16); there is a growing number of evidences showing the lack of close association between apoptotic events and the PPARs action. Some reports indicate that the PPARγ agonists dependent activation of PPARγ might be directly answerable for carcinogenesis, especially, in the colon (17, 18) which renders the physiological role of PPARγ system a very controversial issue.

This report highlights the unexpected consequences of the combined use of the well known and accepted in the literature anti-cancer agents – agonists of PPARγ and γ-radiation. Our results provide evidences for anti-apoptotic action of PPARγ agonists used along with the γ-radiation. Moreover, the up-regulated HSP70, in response to PPARγ agonists and γ-irradiation promotes cell survival.

MATERIALS AND METHODS

Cell culture and irradiation

Caco2 cells were obtained from the American Type Culture Collection (ATCC No. HTB-37). Cells were sub-cultured weekly in MEM containing 4.5 g/l glucose, 10% fetal bovine serum.
(Biochrom GmbH, Germany), 1% non-essential amino acids, penicillin and streptomycin (50 U/ml) (Sigma-Aldrich). Prior to the experiment, 5 × 10⁶ of Caco2 cells were seeded on a 100mm dish in DMEM with the addition of 2% fetal bovine serum to which no antibiotics had been added. We have used for stimulation ciglitazone (CI, 1×10⁻⁶M) and retinoic acid (RA, 1×10⁻⁶M) (Sigma-Aldrich). Some of the cultures 24h after stimulation were transferred in the homeostatic box to the “radiation facilities” and subjected to the γ-irradiation with the therapeutic dose of 2.5Gy of photons using linear accelerator PRIMUS, Siemens. All the experiments were repeated at least three times. The results presented here were taken from the most representative experiments.

**RT-PCR**

Total cellular RNA was isolated using TRIzol Reagent (Gibco-BRL) according to the manufacturer protocol. The synthesis of the first strand cDNA was performed with a Reverse Transcription System (Promega) using 1μg of RNA. For polymerase chain reaction, 2μl of cDNA and oligo primers were used. All PCR reactions were performed with application of Promega PCR reagents. Specific primers, as listed below, were synthesized by Sigma-Genosys (Pampisford, UK). The following human primers were used: β-actin s-5’-AGCGGGAAATCGTGCTGTT-3’, a-5’-GGGTACATGTGTGGTGCCG-3’; bax s-5’-TGGCAGCTGACAGTCTTGAGC-3’, a-5’-GGTCCCAAACCCCGGTCTT-3’; bcl-2 s-5’-CAGATGCACCTGACGCCCTT-3’, HSP70 s-5’-TTTGACACAGCTGGTGAACC-3’, a-5’-GTGAAGATCTGCGTCTGTTGG-3’; PPARγ s-5’-TCTGCGGCCCCAACTTTGGG-3’, as-5’-CTTCAACAGCATGACTCCA-3’.

**Electrophoretic Mobility Shift Assay (EMSA)**

Double-stranded oligonucleotides were prepared from the complementary single-stranded oligonucleotides obtained from Sigma-Genosys (Pampisford, UK) by melting at 95°C for 5 min followed by a cool-down phase of 3 h at ambient temperature. The nuclear extracts (10μg per lane) were examined for band shift with 5 pmol of double-stranded biotinylated heat shock element consensus containing DNA probe (5’-CTAGAAGCTTCTAGAAGCTTCTAGAAA-3’) or consensus PPAR-γ binding element (PPRE) from the acyl-coenzyme A (acyl-coA) oxidase gene (5’-GTCCAGCGGGACCAGGACAAAGGTCAGTTGCGAGTCGAC-3’), obtained from Sigma-Genosys (Pampisford, UK) and labeled with Light Shift Chemiluminescence EMSA kit (Pierce Biotechnology, USA). Complexes were separated by electrophoresis in native 6% polyacrylamide gel and electro-blotted to the Hybond N+ (Amersham Pharmacia Biotech, UK) membrane. After UV cross-linking, the membrane was subjected to procedures in accordance with the manufacturer’s protocol, to detect bands. Unlabelled consensus PPAR-γ binding element (PPRE) probes were used in the competition studies. Unlabelled serum inducible element universal STAT-1, -3 binding probe (5’-AGCTCATTTCCCGTAAATC-3’) was used as a non-competitor.

**Western blot**

Protein extracts from Caco-2 cells were suspended in the immune precipitation buffer. An equal load of protein in each sample was assessed using Quanti Pro BCA Assay Kit (Sigma, USA). Samples containing 5μg of cellular or nuclear proteins were boiled with western-blot sample buffer and loaded on the 12% SDS-polyacrylamide gel. To estimate molecular mass of the separated proteins Page Ruler Protein ladder (Fermentas, Germany) was used. After electrophoresis and transfer of the samples, the PVDF membrane (BioRad, USA) was blocked with blocking buffer (5% non-fat dried milk in PBS) for 1 h at room temperature. This blocking procedure was followed by a 1 h exposure to primary antibody diluted 1:1000 and secondary antibody diluted 1:1000 in blocking buffer. After each antibody probing, the membrane was washed three times for 15 min. in
TBST buffer (0.1M Tris pH 8.0; 0.5M NaCl; 0.5% TritonX-100). Detection of membrane bound proteins was performed using BM Chemiluminescence Blotting Substance (Boehringer Mannheim, Germany). Goat polyclonal anti HSP70 and HSF-1, mouse monoclonal anti PCNA, mouse monoclonal anti PARP or rabbit polyclonal PPARγ antibodies used for western blot were purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

RESULTS

The effect of PPARγ agonists on Caco-2.

Caco-2, a human adenocarcinoma cell cultures (5x10^6 cell per dish) were treated for 24h or 48h with PPAR γ agonists: ciglitazone (CI, 1x10^{-6}M) and retinoic acid (RA, 1x10^{-6}M). Total cellular RNA and proteins (cytoplasmic and nuclear) were isolated 24h or 48h after stimulation. RNA analysis of isolated samples did not show significant differences in the level of mRNA for Bax (Fig.1A II), Bcl-2 (Fig.1A III) or PPAR-γ (Fig.1A V) comparing the un-stimulated control (line 1) to cell treated for 24h (line 2) and 48h (line 3) with mixture of PPAR-γ agonists. Only in case of HSP70 mRNA, in the cell cultures stimulated for 48h (Fig. 1A IV, line 3), substantial increase in transcript abundance in comparison to the control (Fig. 1A IV, line 1) was noticed. Almost the same dynamics of the change was observed for HSP70 protein in the CI/RA stimulated cells during the experiment reaching the highest level at 48h of treatment (Fig. 2B). Although mRNA level for PPAR-γ did not seem to be dependent on CI/RA treatment of Caco-2 cells, PPAR-γ protein expression was

Fig.1. Analysis of Bax, Bcl-2, HSP 70 and PPARγ mRNA expression in the cell cultures stimulated with PPARγ agonists and cultures irradiated with 2.5 Gy of fotons after stimulation. As the reference of the equal load β-actin, the housekeeping gene, was used. Panel A presents the results of PCR reaction for β-actin (I), Bax (II), Bcl-2 (III), HSP70 (IV) and PPAR-γ respectively. Control cells (line 1) were compared with ciglitazone (1x10^{-6}M) and retinoic acid (1x10^{-6}M) stimulated for 24h (line 2) and 48h (line 3) cells with no following γ-irradiation. Panel B represents the result of PCR reaction of mRNA isolated from identically stimulated cells and γ-irradiated 24h after stimulation.
visibly increased in cultures incubated for 24h or 48h with PPAR agonists (Fig. 2A, lines 2, 3) comparing to the control (Fig. 2A, line 1).

To verify the presumption that PPAR-γ agonists act on the level of regulation of transcription EMSA analysis was employed. It revealed increased nuclear translocation and binding of HSF-1 and PPAR-γ in response to CI/RA stimulation. Specific DNA binding of HSF-1 was higher 24 and 48h after stimulation than observed in un-stimulated cultures (Fig. 3A). In case of PPAR-γ 24h period of incubation with its agonist caused massive binding when compared to this observed in the control (Fig. 3B, lines 1, 2). Additional 24h of treatment did not increase much binding of the analyzed nuclear factor in the Caco-2 cultures (Fig. 3B line 3). Specificity of the EMSA reaction was confirmed in non-competitive and competitive test (Fig. 3B, lines nc, cc).

The effect of PPARγ agonists and γ-irradiation on Caco-2.

Cell cultures (5x10⁶ cell per dish) were pretreated for 24h with PPAR γ agonists ciglitazone (CI, 1x10⁻⁶M) and retinoic acid (RA, 1x10⁻⁶M) and part of the cultures were subsequently subjected to γ-radiation (photons) with therapeutic dose of 2,5 Gy. Total cellular RNA and proteins (cytoplasmic and nuclear) were isolated 24h after cultures irradiation or 48h after stimulation in the non irradiated part of experiment to preserve the equal growth time for all samples.

Using PCR it was established that in the irradiated un-stimulated with CI/RA cells mRNA level for Bax (Fig. 1B II, line 1) remained unchanged comparing to the non irradiated cells. Preincubation of the cells with CI/RA almost abolished mRNA level for Bax 48h after stimulation (Fig. 1B II, line 3). On the contrary, mRNA level for Bcl-2 analyzed in the irradiated un-stimulated cell was visibly
increased (Fig. 1B III, line 1) comparing to non irradiated un-stimulated cells (Fig. 1A III, line 1). Preincubation of the cultures with PPAR-γ agonists amplified this phenomenon (Fig. 1B III, lines 2,3). Its worth to notice that CI/RA under the irradiation conditions established previously unobserved anti-apoptotic balance in the Caco-2 cell culture (Fig. 1B II, line 3; Fig. 1B III, line 3). Almost no changes were assigned for HSP70 (Fig. 1B IV) and PPAR-γ (Fig. 1B V) mRNA levels in the γ-irradiation cell comparing to non irradiated cultures. Western blot analysis showed that HSP70 protein cellular abundance, on the contrary to its mRNA level, increased after irradiation in un-stimulated cells (Fig. 4A, line 1). In the presence of CI/RA HSP70 protein level increased slightly (Fig. 4A, line 2). comparing to the un-stimulated γ-irradiated control cells. Irradiation has increased the protein level of PPAR-γ in un-stimulated cells (Fig. 4B, line 1), but this effect was observed only in the cytoplasmic compartment of the cell. In the nucleus PPAR-γ

![Fig. 3. Gel retardation assay for HSF-1 or PPARγ of the nuclear proteins isolated from Caco-2 cells stimulated with PPARγ agonists. As a non-competitor in reaction with control cells nuclear extract unlabelled (line nc) serum inducible element universal STAT-1, -3 binding probe (5'-AGCTCTTCCGTAAATC-3') was used. As a competitor unlabelled consensus PPAR-γ binding element (PPRE) probe in reaction with control cells nuclear extract was used (line cc). Panel A: Analysis of HSF-1 binding in nuclear extracts of control cells (line1), stimulated with ciglitazone (2x10^-6M) and retinoic acid (1x10^-6M) for 24h (line 2) and 48h (line3) . Panel B: Analysis of PPARγ binding in nuclear extracts of control cells (line1), stimulated with ciglitazone (1x10^-6M) and retinoic acid (1x10^-6M) for 24 h (line 2) and 48h (line 3).]
protein was undetectable in un-stimulated as well as CI/RA stimulated cells (Fig. 4C, line 1, 2). Lack of nuclear translocation and DNA binding of the PPAR-γ in irradiated cultures was confirmed using EMSA test (Fig. 5).

The effect of PPARγ agonists and γ-irradiation on apoptosis in Caco-2 cell cultures.

Our primary objective was to find the best proportion of PPARγ agonists in combination with γ-radiation to induce apoptosis in colon carcinoma cells. Surprisingly, we failed to find any signs of apoptosis in the cultures subjected to the stimulation with PPARγ agonists followed by γ-radiation as well as stimulation alone. The results of TUNEL and DNA ladder tests were negative.
The results of PCR analysis for Bax and Bcl-2 were inconclusive. The sole result suggesting the possible pro-apoptotic changes in our cultures was revealed using western blot analysis of PARP. g-irradiated cells manifested typical for apoptosis pattern of PARP degradation showing both the native 112 KD and digested 85 KD forms (Fig. 6B line 1). Stimulation with PPAR\(\gamma\) agonists prior to the irradiation decreased almost completely elimination altogether the process of PARP degradation (Fig. 6D line 2). No signs of PARP digestion were found in the cell cultures stimulated but not irradiated (Fig. 6A).

**DISCUSSION**

Peroxisome proliferator-activated receptors, nuclear receptors that regulate lipid and glucose metabolism, were shown to play a beneficial role in treating neoplasm. Over last few years, the significant number of authors presented evidences for anti-tumor activity of PPAR\(\gamma\) in different types of cancer. Its agonists, members of the thiazolidinedione family (TZD) like troglitazone and ciglitazone, were suggested to mediate anti-proliferative effects on cancer cell lines including those of prostate (19), breast (20), colon (21), thyroid (22), lung...
(23), and pituitary carcinoma (24). Proposed mechanisms of anti tumor activity were mostly PPARγ independent involving inhibition of Bcl-XL and Bcl-2 function by blocking BH3 domain (25), down-regulation of cyclin D1 expression causing cell-cycle arrest (26, 27) or up-regulation of the expression of the CDK inhibitor p21CIP1 (28, 29). All the mechanisms mentioned above clearly demonstrated the dissociation of PPARγ activation from triggering apoptosis in cancer cells.

On the other hand, several lines of evidences suggest that activators of PPARγ enhance colon polyps formation (30) and promote the development of colon tumors (17, 18). All these conflicting information convince us that the precise mode of PPARγ activators action is mostly unknown and requires further elucidation.

From among different strategies for neoplasm treatment, radiotherapy seems to be one of the most radical. It is known to be useful in the tumor mass reduction due to the induction of apoptotic cell death. Combined with chemotherapy is classified as the basic treatment of majority of colorectal tumors. Nevertheless, some tumor characteristics might attenuate or even abolish its beneficial effect. One of these conditions is the high level of HSPs expression. The overexpression of HSP 27 and/or HSP 70 has been associated with metastasis, poor prognosis and resistance to radio- as well as chemotherapy (8, 9). The most probable explanation of this phenomenon is the selective prevention of caspase 9 and caspase 3 processing by HSP 70 (31). Some authors suggest that the inhibition of caspases may not be the sole mechanism behind the survival effect of HSP 70 (32).

We have presented above the evidence that PPARγ under influence of its agonists was translocated to nucleus and bind to the specific DNA sequence (Fig. 6). Western blot analysis of PARP-1 proteolysis products in the cellular extracts of the cell cultures after stimulation with PPARγ agonists and followed by γ-irradiation. Panel A presents results of proteolysis of PARP-1 in the cell cultures stimulated with PPARγ agonists or stimulated with PPARγ agonists prior to the γ-irradiation (panel B). Lines 1, 2 indicate control cells and stimulation with retinoic acid (1x10^-6M) and ciglitazone (1x10^-6M) respectively.
3B). At the same time, we have observed PPARγ agonists dependent nuclear translocation of HSF-1 (Fig. 3A) followed by the increased expression of HSP 70 (Fig. 2B) which is consistent with the result of Konturek et al (33).

γ-irradiation of the cells abolished nuclear translocation of PPARγ under its agonists treatment (Fig. 5) and preserved PPARγ in the cytoplasmic pool (Fig. 4C). But it did not affect the HSP 70 expression in response to ciglitazone and retinoic acid (Fig. 4A). Upon the basis of our results and encouraged by the literature data, we would like to propose the mechanism of anti-apoptotic action of activated PPARγ in γ-irradiated human colon cancer cells. We postulate that γ-irradiation restrained PPARγ in cytoplasmic pool (Fig. 4B,C) precluding its proper action in response to agonists (Fig. 5). It prevents any pro-apoptotic effect of PPARγ in colon cancer cells. Undisturbed by the γ-irradiation PPARγ agonists dependent HSP 70 activation renders the cells resistant to radiotherapy (Fig. 4A). That even diminishes susceptibility to apoptosis of Caco2 manifested by PARP degradation (Fig.6B). Moreover, combined γ-irradiation and CI/RA treatment of the cells changed the equilibrium between Bax and Bcl-2 mRNA to anti apoptotic state with increased expression of Bcl-2 and almost abolished expression of Bax (Fig. 1B II, line 3; Fig. 1B III, line 3). This report highlights the unexpected consequences of the combined use of the well known and accepted in the literature anti-cancer agents – agonists of PPARγ and γ-radiation. Our results provide evidences for anti-apoptotic action of PPARγ agonists used along with the γ-radiation but, the exact molecular mechanism of the observed phenomenon in colorectal cancer cells needs further studies.

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