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Pioglitazone Inhibits Growth of Carcinoid Cells and Promotes TRAIL-Induced Apoptosis by Induction of p21$^{waf1/cip1}$

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Key Words

Pioglitazone · Carcinoid · TRAIL · Apoptosis · p21$^{waf1/cip1}$

Abstract

Background/Aims: We investigated the effect of the peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist pioglitazone on growth and TRAIL-induced apoptosis in carcinoid cells. ***Methods:*** Carcinoid cells were incubated without and with pioglitazone. Effects on growth were examined by cell count and cell cycle analysis. p21$^{waf1/cip1}$ expression was determined by Western blotting. Cytotoxicity assay was performed by FACS analysis. ***Results:*** Pioglitazone suppressed the growth and induced apoptosis of carcinoid cells. Additionally, pioglitazone significantly enhanced carcinoid cell death induced by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). The enhancement of TRAIL-induced apoptosis was associated with an upregulation of cyclin-dependent kinase inhibitor p21$^{waf1/cip1}$ in pioglitazone-treated carcinoid cells. Importantly, overexpression of p21$^{waf1/cip1}$ in carcinoid cells by adenoviral gene transfer of p21 sensitized them to TRAIL-induced apoptosis. ***Conclusions***: These results suggest that pioglitazone inhibits cell growth and sensitizes cells to TRAIL-induced apoptosis by induction of p21$^{waf1/cip1}$. Therefore, pioglitazone can be an effective therapeutic adjuvant for the treatment of carcinoid tumors.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a newly identified member of the TNF family [1]. It shares the highest sequence homology with Fas (CD95, Apo-1) ligand, another member of the TNF family that is capable of inducing apoptosis. However, in contrast to Fas ligand (FasL), TRAIL induces apoptosis of a variety of tumor cells, but not normal cells [2]. To date, five different receptors for TRAIL have been identified: i.e. Death Receptor 4 (DR4, TRAIL-R1), Death Receptor 5 (DR5, TRAIL-R2), Decoy Receptor 1 (DcR1, TRAIL-R3, TRID), Decoy Receptor 2 (DcR2, TRAIL-R4, TRUNDD), and osteoprotegerin. Binding of TRAIL to either DR4 or DR5 recruits FADD to its intracellular death domain and activates the caspase cascade resulting in cell death [3, 4]. DcR1 and DcR2 are decoy receptors that do not transduce apoptotic signals but may protect cells from TRAIL-induced apoptosis [5–8]. Osteoprotegerin is the only known soluble receptor for TRAIL, which has been reported to regulate osteoclastogenesis [9].

Pioglitazone is an antidiabetic thiazolidinedione that specifically activates the nuclear transcription factor PPAR-γ. PPAR-γ exists in two isoforms, PPAR-γ1 and PPAR-γ2 [10]. While PPAR-γ2 is expressed primarily in adipocytes, PPAR-γ1 has been identified in a variety of tissues [10, 11]. It has been reported that PPAR-γ plays

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important roles in lipid metabolism and inflammation [10] and that activation of PPAR-γ induces apoptosis of differentiated macrophages and macrophages activated by TNF-α or interferon-γ [12]. We have recently reported that PPAR-γ regulates TRAIL-induced apoptosis of Jurkat T cells and colon cancer cells (HT-29 and SW480) [13]. In this report, we studied the effect of pioglitazone on the growth and apoptosis of carcinoid cells, and discovered a novel role for cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ in TRAIL-induced apoptosis. Treatment of endocrine tumors is extremely challenging because they are resistant to chemotherapy and radiation and show only a limited sensitivity to hormones and cytokines such as somatostatin or interferon-α [14]. Data reported here suggest that a combination of TRAIL and pioglitazone may be effective in treating these tumors.

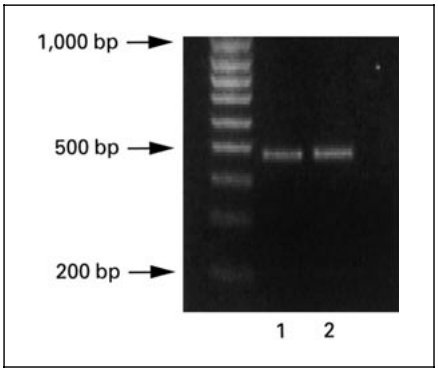


Fig. 1. PPAR-γ expression in NCI-H727 carcinoid cells and HT-29 colon cancer cells. Total RNA was extracted and RT-PCR was performed as described in 'Methods'. PPAR-γ expression (474 bp) in NCI-H727 and HT-29 cells is shown in lanes 1 and 2, respectively.

Materials and Methods

Cell Lines and Reagents

NCI-H727 carcinoid cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Pioglitazone (Takeda, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture to a final concentration of 20 μ*M*. Control cultures received the same amount of DMSO (which was less than 0.2% of the medium).

RT-PCR

Total RNA was extracted from cells using the RNeasy kit, and reverse transcribed to cDNA using the Omniscript cDNA synthesis kit with hexamer primers (Quiagen, Valencia, Calif., USA) per manufacturer's instructions. For detection of PPAR-γ mRNA, the following primers were used: Sense primer, 5′-TCTCTCCGTAATGGAAGACC-3′, and antisense primer, 5′-GCATTATGAGACATCCCCAC-3′ [15]. PCR amplification was performed using Taq polymerase (Promega, Madison, Wisc., USA) under the following conditions: Denaturation at 95°C for 2 min, amplification for 35 cycles (95°C for 45 s, 55°C for 1 min and 72°C for 1 min), and extension at 72°C for 7 min. The PCR products were then separated by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide.

Cell Cycle Analysis

Cells were cultured in 75 cm^2 cell culture flasks with or without 20 μ*M* pioglitazone for 4 days. Cells were then harvested, and fixed in ice-cold 70% (v/v) ethanol for 24 h at 4°C. After centrifugation (2,000 *g*, 4°C, 4 min), the cell pellet was resuspended in phosphate-buffered saline (PBS) (pH 7.4) containing 1 μg/ml of DNase-free RNase, 50 μg/ml of propidium iodide (PI) and 0.1% glucose. Cells were incubated at room temperature in the dark for 4 h, and DNA content was determined by flow cytometry using a FACScan flow cytometer (Becton-Dickinson, San Jose, Calif., USA). Histograms were analyzed using the ModFit 2.0 software (Verity Software House, Topsham, Me., USA).

Cytotoxicity Assay

Cells were pre-incubated in 24-well plates with or without pioglitazone for various times followed by an incubation with or without recombinant human TRAIL (Biomol, Plymouth Meeting, Pa., USA) for 16 h at 37°C. Cells were then harvested, washed and resuspended in PBS (pH 7.4) containing 1 m*M* glucose and 2 μg/ml of propidium iodide. PI fluorescence intensity was determined by flow cytometry using a FACScan flow cytometer.

Western Blot Analysis

Cell lysates were first separated by polyacrylamide gel electrophoresis. The $p21^{waf1/cip1}$ protein was then detected by Western blotting [16] using an anti-$p21^{waf1/cip1}$ antibody (clone SXM30) from Pharmingen (San Diego, Calif., USA).

Results

Pioglitazone Inhibits Growth of Carcinoid Cells by Blocking Cell Cycle Progression

PPAR-γ is constitutively expressed in a variety of cell types [17, 18]. To determine whether PPAR-γ is expressed in carcinoid cells, we tested its gene expression in NCI-H727 cells by PCR. As shown in figure 1, PPAR-γ mRNA is readily detectable in NCI-H727 cells as well as HT-29 colon cancer cells which are known to express high levels of PPAR-γ mRNA [19].

To determine the effect of PPAR-γ activation on the growth of NCI-H727 carcinoid cells, we treated them with 20 μ*M* pioglitazone for up to 8 days. As shown in figure 2a, treatment with pioglitazone significantly inhibited the proliferation of carcinoid cells. This was associated

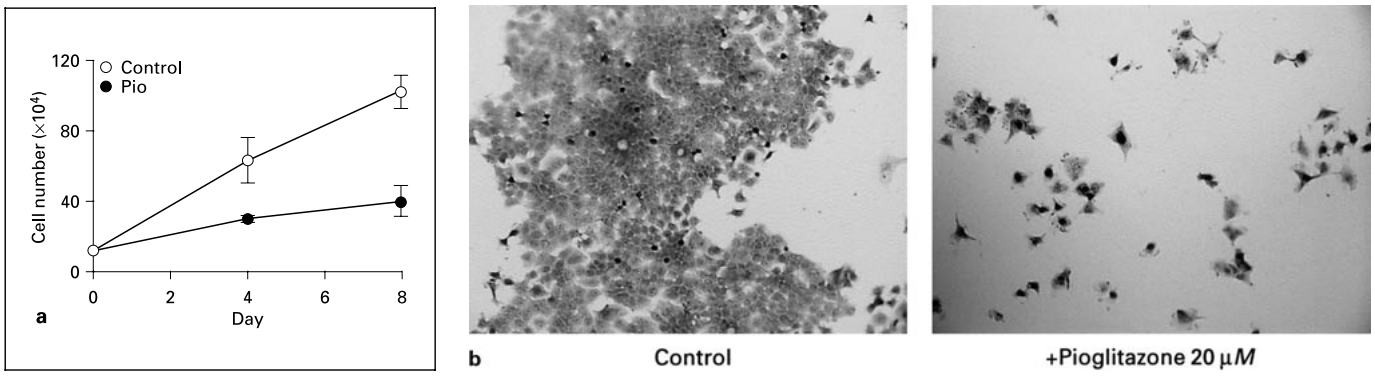


Fig. 2. a Pioglitazone inhibits growth of NCI-H727 carcinoid cells. 1.25×10^4 cells were cultured with or without 20 μ*M* pioglitazone for up to 8 days. The number of cells in each culture was determined using a Rosenthal counting chamber. Data presented are means of duplicated cultures of a representative experiment. **b** Pioglitazone inhibits cell growth and prevents colonization of NCI-H727 cells. NCI-H727 cells were cultured with or without 20 μ*M* pioglitazone for 4 days, stained with hematoxylin/eosin, and examined by light microscopy. Original magnification ×200.

with a reduced ability of these cells to colonize (fig. 2b). Further analysis of these cells revealed significant blockade of cell cycle progression by pioglitazone (fig. 3). Specifically, pioglitazone increased the percentage of cells in the G0/G1 phase while it decreased the percentage of cells in the S phase of the cell cycle (fig. 3).

Pioglitazone Enhances TRAIL-Induced Apoptosis of Carcinoid Cells

To determine whether PPAR-γ plays any role in TRAIL-induced apoptosis of carcinoid cells, we pretreated NCI-H727 cells with 20 μ*M* pioglitazone for 4 days and then examined their sensitivity to TRAIL-induced apoptosis. As shown in figure 4, TRAIL alone induced moderate apoptosis of these cells, suggesting that they are sensitive to TRAIL-induced apoptosis. Pretreatment of these cells with pioglitazone significantly increased their sensitivity to TRAIL-induced apoptosis, with the percentage of apoptotic cells increased by 3-fold in the pioglitozone-treated group (fig. 4). Pioglitozone alone also induced detectable apoptosis of carcinoid cells, which is consistent with reports of the effect of PPAR-γ agonists on other cell types [15, 20].

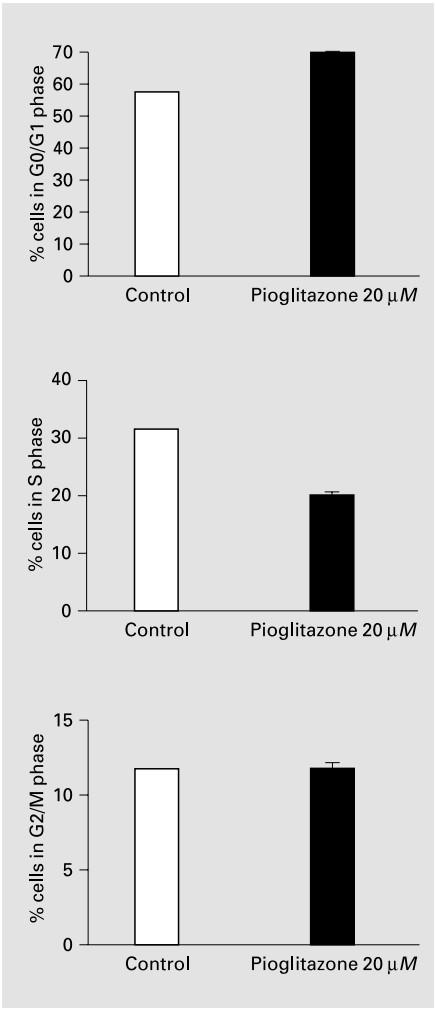
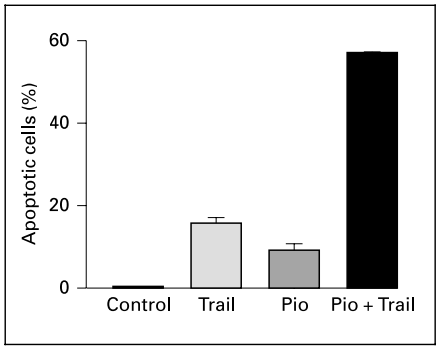


Fig. 3. Effect of pioglitazone on cell cycle progression in NCI-H727 cells. NCI-H727 cells, 10^6 cells/ml, were cultured with or without 20 μ*M* pioglitazone for 4 days. Cells were then harvested, fixed in 70% ethanol, and stained with propidium iodide. The percentage of cells in each phases of cell cycle was determined by flow cytometry using the ModFit 2.0 software.



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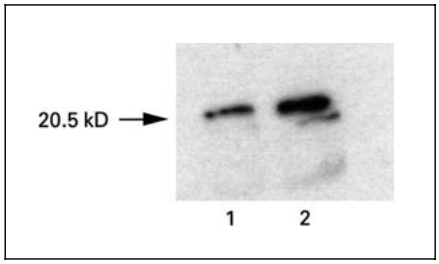

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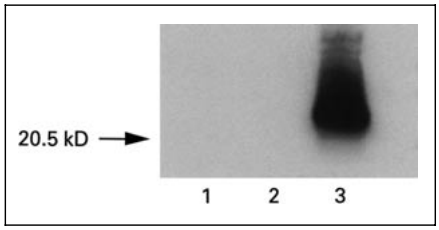

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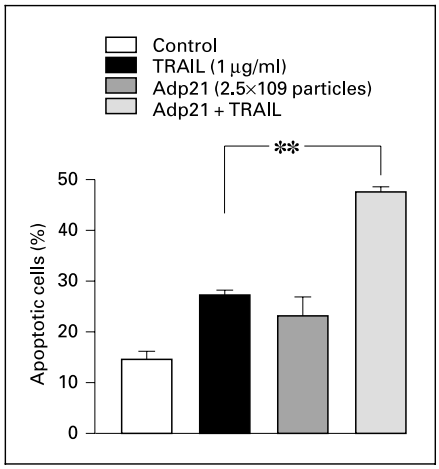

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Fig. 4. Pioglitazone enhances TRAIL-induced apoptosis of NCI-H727 cells. NCI-H727 cells, 5×10^5 cells/ml, were cultured with or without 20 μ*M* pioglitazone for 4 days. Cells were then treated with 200 ng/ml of TRAIL for 16 h. The percentage of apoptotic cells was determined by flow cytometry as described in Methods. Data presented are means ± SEM of the percentages of apoptotic cells pooled from two experiments. Control cells were treated with vehicle alone. Pio = Pioglitazone.

Fig. 5. Pioglitazone induces $p21^{waf1/cip1}$ expression in NCI-H727 cells. NCI-H727 cells, 10^6 cells/ml, were cultured with 20 μ*M* pioglitazone for 4 days. Proteins were extracted and $p21^{waf1/cip1}$ protein was detected by Western blot analysis. Each lane was loaded with 50 μg protein. Lane 1 = Cells treated with vehicle; lane 2 = cells treated with pioglitazone.

Fig. 6. Expression of $p21^{waf1/cip1}$ in NCI-H727 cells infected with $Adp21^{waf1/cip1}$. NCI-H727 cells, 10^6 cells/ml, were infected with 2.5×10^9 particles of $Adp21^{waf1/cip1}$ (lane 3). Control cells were either noninfected (lane 1) or infected with 2.5×10^9 particles of AdLacZ (lane 2). Proteins were extracted and $p21^{waf1/cip1}$ was detected by Western blot analysis. Each lane was loaded with 50 μg protein.

Fig. 7. Overexpression of $p21^{waf1/cip1}$ enhances TRAIL-induced apoptosis. NCI-H727 cells, 10^6 cells/ml, were either infected or not infected with 2.5×10^9 particles of $Adp21^{waf1/cip1}$. 24 h later, cells were cultured with or without TRAIL (1 μg/ml) for an additional 16 h. The percentage of apoptotic cells was determined by flow cytometry as described in 'Methods'. Data presented are means ± SEM of percentages of apoptotic cells pooled from two experiments. ** $p < 0.005$ as determined by ANOVA.

$p21^{waf1/cip1}$ Expression Promotes TRAIL-Induced Apoptosis in Carcinoid Cells

Recently, it was reported that the PPAR-γ agonist troglitazone upregulates the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ in several tumor cell lines [21]. However, the role of $p21^{waf1/cip1}$ in apoptosis is unclear. Several reports suggest that $p21^{waf1/cip1}$ may prevent cell death [22–24]. On the other hand, Fas (CD95) ligation in T cells induces expression of $p21^{waf1/cip1}$ and promotes Fas-induced apoptosis [25].

To explore the potential roles of $p21^{waf1/cip1}$ in TRAIL-induced apoptosis and its regulation by PPAR-γ, we examined the effect of PPRA-γ on $p21^{waf1/cip1}$ expression and its impact on TRAIL-induced apoptosis. As shown in figure 5, $p21^{waf1/cip1}$ expression was significantly increased in carcinoid cells treated with 20 μ*M* pioglitazone. This may explain the cell cycle arrest induced by pioglitazone as shown in figure 3. To directly examine the effect of upregulated $p21^{waf1/cip1}$ expression on TRAIL-induced apoptosis, we infected carcinoid cells with a recombinant adenovirus carrying the $p21^{waf1/cip1}$ gene. As shown in figure 6, expression of $p21^{waf1/cip1}$ was dramatically increased in cells infected with $p21^{waf1/cip1}$ virus, but not the control virus. Since the exposure time of the film was only 10 s, basal expression of p21 was not detectable in controls. Figure 5 shows that basal p21 expression in controls

is detectable after an exposure time of 1 min. Importantly, overexpression of $p21^{waf1/cip1}$ significantly increased the sensitivity of these cells to TRAIL-induced apoptosis (fig. 7).

Discussion

TRAIL is a newly described member of the TNF superfamily, which shares the highest homology with FasL. However, unlike FasL, TRAIL selectively kills transformed tumor cells, but not most nontransformed normal cells. Putative mechanisms responsible for the TRAIL resistance include the expression of TRAIL decoy receptors on the cell surface and/or expression of anti-apoptotic genes such as FLIP, IAP or survivin in resistant cells [26–28]. Expression of anti-apoptotic or survival genes may be regulated by transcription factors such as NF-κB and PPAR-γ [29]. We have previously reported that TRAIL-induced apoptosis in Jurkat cells and colon cancer cells is regulated by these transcription factors. Activation of the nuclear receptor PPAR-γ or inhibition of NF-κB resulted in an increased sensitivity of cells to TRAIL-induced apoptosis [13]. Encouraged by these results and other studies demonstrating a pro-apoptotic effect of thiazolidinediones on tumor cells [15, 20], we investigated the effect and the mechanisms of action of a PPAR-γ agonist in TRAIL-induced apoptosis using a carcinoid cell line, NCI-H727. NCI-H727 cells were originally derived from a lung carcinoid tumor and are the best-characterized carcinoid cells. We found that pioglitazone inhibits the growth of NCI-H727 cells by blocking G1-to-S phase cell cycle progression and sensitizes them to TRAIL-induced apoptosis. Furthermore, we demonstrated that pioglitazone upregulates the expression of cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, which in turn is capable of sensitizing carcinoid cells to TRAIL-induced apoptosis. To our knowledge, this is the first report on the role of $p21^{waf1/cip1}$ in TRAIL-induced apoptosis.

Several reports suggest that thiazolidinediones alone are capable of inducing apoptosis of tumor cell lines [15, 20]. Although the precise pro-apoptotic actions of these substances remain to be elucidated, PPAR-γ-mediated inhibition of NF-κB has been suggested to be involved [13, 30]. Results reported here indicate that the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ may also play an important role in this process. $p21^{waf1/cip1}$ is involved in multiple biological functions including the inhibition of cyclin-dependent kinases, which results in suppression of cell cycle progression [31]. However, the roles of $p21^{waf1/cip1}$ in apoptosis are poorly understood. Previous reports suggest that $p21^{waf1/cip1}$ may play an anti-apoptotic role by inhibiting cyclin-dependent kinases, or by regulating the functions of caspases, stress-activated kinases, or survival genes [32]. On the other hand, it has also been shown that overexpression of $p21^{waf1/cip1}$ promotes apoptosis of BHK21 cells [33]. Our results suggest that $p21^{waf1/cip1}$ expression enhances TRAIL-induced apoptosis in carcinoid cells. This is consistent with a recent report that $p21^{waf1/cip1}$ promotes Fas-induced apoptosis in T cells [25]. However, it is unclear whether the apoptotic activity of $p21^{waf1/cip1}$ is resulted from its effect on cell cycle. In this regard, it has been reported that although the transcription factor E2F regulates both cell cycle and apoptosis [34, 35], the two functions are not mutually dependent on each other [36]. Since $p21^{waf1/cip1}$ has been shown to directly interact with the transcription factor E2F [37], it is conceivable that it may promote the apoptotic effect of the E2F while inhibit its effect on cell cycle.

Treatment of neuroendocrine tumors remains extremely challenging. In the case of carcinoid tumors, surgery is rarely curative and external radiation therapy has not been successful [14]. Similarly, carcinoid tumors respond poorly to chemotherapy with only a small fraction of them responding to streptozotocin, doxorubicin or 5-fluorouracil [38, 39]. Somatostatin analogues inhibit the release of peptide hormones from neuroendocrine tumors attenuating clinical symptoms. However, tumor reduction is observed only in less than 10% of patients treated with somatostatin analogues [40, 41]. Finally, although 50% of the patients with carcinoid tumors respond to interferon-α therapy, only about 15% of them show significant tumor shrinkage [41]. Data reported here suggest that pioglitazone inhibits growth of carcinoid cells and sensitizes them to TRAIL-induced apoptosis. Thus, a combination of pioglitazone and TRAIL may be an effective therapeutic regimen for the treatment of carcinoid tumors.

Acknowledgements

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