PPARγ Agonist, Pioglitazone, Suppresses Melanoma Cancer in Mice by Inhibiting TLR4 Signaling

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ABSTRACT – Background: Although previous studies demonstrated an anticancer effect for the ligands of peroxisome proliferator-activated receptor gamma (PPARγ) through activation of its anti-inflammatory responses, nevertheless the anti-tumor mechanism of PPARγ has not been intensively investigated. One of the molecules involved in cancer progression is toll-like receptor 4 (TLR4). TLR4 signaling induces NF-κB activity which has a crucial role in cancer progression. In this study, we examined the cross-talk between PPARγ and TLR4 in the melanoma.

Methods: B16F10 melanoma cells were cultured with or without LPS for 24 hr. The cells were subcutaneously injected to two groups of C57BL/6 mice. After the development of palpable tumors each group of animals were divide to four sub-groups and received pioglitazone in different dose ranges (0,10,50,100 mg/kg/day) for 10 days. At the end of the study, the expression of Tlr4, Myd-88, Nf-kb1 genes was evaluated by qRT-PCR in different groups in mice tumor. The TLR-4 protein expression was evaluated by IHC. TNF-α level in mice tumor and serum were measured by ELISA kits. Tumor volume was measured with Vernier calipers. Results: We observed that activation of PPARγ by its agonist, pioglitazone, reduces tumor volume, Tlr-4, Myd-88, Nf-kb1 mRNA expression, TLR4 protein expression and TNF-α production in melanoma tumor especially in groups that were injected with LPS -stimulated cells. Moreover, treatment of melanoma cells with pioglitazone showed that the inhibitory effects of pioglitazone on LPS-induced inflammatory responses were TLR4 dependent. Conclusion: The results indicate that pioglitazone, a PPARγ agonist, has a beneficial protective effect against melanoma via interfering with the TLR4-dependent signaling pathways.

INTRODUCTION

Toll-like receptors (TLRs) are major components of the innate immune system that recognize the conserved molecular structures of pathogens. There are many different cell types, ranging from epithelial to cancer cells that express TLRs (1). TLR4, one member of the TLRs family, could serve as important regulators in the development of a variety of cancers such as melanoma (2). TLR4 can be expressed by melanocytes, which are the main cell types that can become melanoma (3).

Previous findings suggest that over-expression of TLR4 by melanoma cells triggers an inflammatory response leading to tumor development (4). Previously we have shown that TLR4 Stimulation with a specific ligand( lipopolysaccharides: LPS) up regulates TLR-4 expression and activates Myd-88 and Nf-kb in melanoma cells , that are three major factors in TLR4 signaling pathway (5). The TLR signaling adaptor MyD88 plays an important role in tumor development. Overexpression of MyD88 was described in several tumors(6)and enhanced levels of MyD88 were associated with poor prognosis in some cancers(7–9). MyD88 is an essential adaptor molecule for induction of inflammatory cytokines through NF-κB triggered by TLR4(10).It is well established that LPS elicit inflammation via increasing levels of inflammatory cytokines (11). One of these cytokines, tumor necrosis factor-α (TNF-α), has been reported to up regulate malignant melanoma invasion and migration in vitro (12).

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(PPARγ) is a ligand-activated transcription factor, which is involved in melanoma cell proliferation (13), differentiation (14), apoptosis (15) and angiogenesis (16). Pioglitazone, a PPARγ agonist, significantly reduces inflammation through inhibition of NF-κB activity (17).

Recent evidence suggests that PPARs and TLRs signaling pathways have crosstalk in different diseases (18). The data reveal a regulatory feedback loop in which PPARγ represses NF-κB-mediated inflammatory signaling via TLR4 pathway (19). Recently, a study has shown that PPARγ activation with its specific agonist inhibits proliferation and enhances the apoptosis of esophageal cancer cells by affecting the TLR4-dependent MAPK pathway (20).

The studies provide evidence that PPARγ is implicated in the regulation of NF-κB signaling by modulating TLR4 expression and TNF-α production (11,21–24). Recently we showed that fenofibrate, a PPARα agonist; inhibit melanoma progression via interaction with TLR4 signaling pathway (25). Also our recent in vitro study has shown that pioglitazone inhibits TLR4 signal transduction pathway in melanoma cancer. As we know because the result of in vitro studies can be different from in vivo because of this, in vitro study is done in a controlled environment outside of a living organism and in vitro cell culture is a significantly different environment from that of a solid tumor.

So, we investigated the effects of PPARγ agonist pioglitazone on LPS mediated inflammation and the TLR4 signaling pathway involved in melanoma cancer in vivo to clarify the potential mechanisms of the anti-inflammatory action of pioglitazone.

MATERIALS AND METHODS

Cell and Reagents

The mouse melanoma cell line (B16F10) was obtained from the National Cell Bank of Iran (affiliated to Pasteur Institute, Tehran, Iran). Ultrapure LPS-EB from E. coli 0111: B4 was provided by InvivoGen (San Diego, CA, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from Gibco BRL (Carlsbad, CA, USA). Pioglitazone was produced by Sigma (St. Louis, MO, USA). Mouse TNF-α ELISA kit was purchased from eBioscience (San Diego, CA, USA). TLR4 antibody (mouse monoclonal, sc-293072 HRP) was obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, TX, USA). For drug preparation, pioglitazone was dissolved in polyethylene glycol.

Cell culture and Treatment

The B16-F10 cells were grown in DMEM supplemented with normal FBS. One day before the cell injection to animals, cells were divided into two groups: one group was treated with LPS (5µg/ml) for 24hr and the other group was treated with complete medium only. After incubation the cells were harvested and cell densities were measured using a haemocytometer after the addition of trypan blue.

Animals

C57BL/6 male mice (n = 48, 6-8 weeks old) were purchased from the Pasture Institute of Iran (Tehran). All mice were housed in standard cages with standard with 22-23°C temperature and 12/12 dark/light cycle. They were maintained one week for acclimatization and then weighted once a week during the experiment. All the experiments were approved by the Ethical Committee of Isfahan University of Medical Sciences Isfahan, Iran (approval ID: IR. MUI . REC.1394.3.617).

After incubation of cells with or without LPS, tumors were induced into two groups (each one was 24 mice) and injecting 1×10⁶ cells in 200 µL of PBS subcutaneously in the right back flank (26). The day of cell transplantation was designated as day 0. Seven days later, each main group of mice with a palpable tumor were randomly distributed into four subgroups (each group contain six mice). Four LPS groups received an intraperitoneal injection of pioglitazone in different dose ranges (0,10,50,100 mg/kg/day) for 10 days. Their control group received only vehicle (normal saline &Polyethylene glycol). The other four groups that were injected with untreated cells were received the same pioglitazone treatment and their control group was administrated vehicle for 10 days. Their control group received only vehicle (normal saline &Polyethylene glycol). The other four groups that were injected with untreated cells were received the same pioglitazone treatment and their control group was administrated vehicle for 10 days. All eight groups of animals were euthanized at the study endpoint (20 days) and the tumors were excised for further examination.

Tumor volume

The tumor volume was calculated by determining the length and width of the tumor as measured using
Vernier calipers. Tumor volumes based on caliper measurements were calculated by the modified ellipsoidal formula(27).

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\text{Tumor volume} = \frac{1}{2}(\text{length} \times \text{width}^{2})
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**Total RNA isolation**

Tumor tissues obtained from each mouse were frozen in liquid nitrogen for RNA extraction. Tumor tissues were homogenized in Lysis Buffer (Thermo Scientific) using Micro Smash MS-100R (Tomy Digital Microbiology Co Ltd, Tokyo, Japan) and RNA was extracted by using total RNA Extraction Kit GeneJet RNA Purification kit (Thermo Scientific, (EU) Lithuania) according to the manufacturer’s instructions.

The concentrations and quality of RNA were determined by a spectrophotometer (BioTek Instruments, Epoch, USA) and gel electrophoresis. RNA samples were treated with RNase free DNase (Qiagen, USA) to eliminate the genomic DNA. Total RNA from each sample was reverse-transcribed into cDNA by using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania).

**Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)**

Real-time qRT-PCR was performed for the detection of the mRNA expressions of Tlr-4, Myd-88, and Nf-kb1. The primer sequences for Tlr-4, Myd-88, Nf-kb1, and beta-actin were designed from the sequence list of GeneBank database (National Centre for Biotechnology Information, NCBI) using Beacon designer 8 software and then blasted against GeneBank database sequences (Tlr-4, 5′-AGTGGCTGGATTTATCCAGGTGTG-3′(forward) and 5′-TTGAGAGGTGGTGTAAGCCATGCC-3′(reverse) ; Myd-88, 5′-AAGTCTAGGAAGGCCCAAA-3′ (forward) and 5′-CTGGGGAGAAAACAGCTGAG-3′ (reverse) ; Nf-kb1, 5′-ACACGAGGCTACAACATCTGTG-3′(forward) and 5′-GTTACCAGAGACCTCAT-3′(reverse) ; beta-actin, 5′-GCTGTATTCCCCCTCCATCGT-3′(forward) and 5′-CAGGTTGTCGTT-3′(reverse)).

Real-time RT-PCR was carried out by the Maxima SYBR Green Rox qPCR master mix kit (Fermentas, Vilnius, Lithuania). Real-time PCR reactions were performed using Corbett machine, Rotorgene 6000 (Australia). The PCR amplification conditions consisted of 15 min at 95 °C followed by 40 cycles of the denaturation step at 95 °C for 15s and annealing and extension for 1 min at 60 °C.

The expression level of each target gene normalized with respect to the expression of housekeeping beta-actin gene was calculated as 2\(^{-\Delta\Delta Ct}\). These experiments were carried out in triplicate and repeated independently at two times.

**Immunohistochemistry**

Tumor tissues were fixed in 10% formalin, embedded in paraffin, sectioned and immunohistochemistry performed as described previously(25). Finally, images were captured using the Leica microscope equipped with a Leica camera (DFC450 C). The TLR-4 protein expression was evaluated by ImageJ software.

**Enzyme-linked immunosorbent assay (ELISA)**

For determination of TNF-α level in tumor lysate, snap frozen tumors were lysed using RIPA buffer containing a protein inhibitor cocktail (Calbiochem, San Diego, CA). Tissue lysates were cold centrifuged at 12,000rpm for 30min and then the supernatants were collected. Protein concentration was determined by the Bradford method using Bio-Rad Protein Assay Dye and microtiter plate reader (ELX 800-BioTek-USA). Finally level of TNF-α was measured by Elisa kits in tumor lysate.

To detect the effect of pioglitazone on TNF-α level in mouse serum, concentrations of TNF-α was determined in serum aliquots from animals of the different groups with commercially TNF-α Detection ELISA Kits (Mouse TNF- α Instant ELISA and mouse) according to the manufacturer’s instruction.

**STATISTICAL ANALYSIS**

Data were expressed as means ± S.E.M. Differences among more than two groups were determined either by one-way ANOVA followed by post hoc Dunns multiplecomparison test. Differences between two groups were tested by t-tests. The results analyzed for gene expression with one sample t-test between the treated and the control groups and with one-way ANOVA between the other groups followed by Tukey’s test. A value of P < 0.05 was considered statistically. Statistical analysis was performed using the SPSS 19 software.
RESULTS

Pioglitazone inhibits the growth of subcutaneous B16F10 tumors

At first, to determine the antineoplastic effect of pioglitazone in vivo, male C57BL/6 mice were used. As soon as tumor became palpable, about 7 days after cell injection, mice received subcutaneous injection of vehicle or pioglitazone (0, 10, 50, 100 mg/kg/day) and continued on diets for 10 more days. Untreated control mice rapidly developed visible tumors and dramatic growth was observed throughout the course of the study. In contrast, treatment of mice with pioglitazone with 50 (p<0.05) and 100 (P<0.01) mg/kg/day concentration significantly attenuated the ability of cells to develop tumors. These data clearly demonstrate that pioglitazone has anti-melanoma activity in vivo.

To examine the inhibitory effect of pioglitazone on LPS-stimulated tumor growth, before cell injection to the mice, B16f10 cells were pretreated with 5µg/ml of LPS for 24. As demonstrated in Figure 1, administration of pioglitazone led to a significant reduction in tumor volume in 10 and 50 mg/kg/day doses (P<0.05) compared to the mice that received only LPS treated cells.

Pioglitazone decreases expressions of Tlr4, Myd-88, and Nf-kb1 in mice tumor

To assess the effect of LPS on TLR4 signaling in melanoma tumor, we measured the amount of Tlr4, Myd-88 and Nf-kb1 mRNA expression in mice that were injected with the pre-treated B16F10 cells with or without LPS for 24 h. Treatment of cells with LPS before injection to mice significantly elevated the levels of Tlr4 (P<0.001), Myd-88 (P<0.01) and Nf-kb1 (P<0.001) mRNAs in tumor tissue. We also determined whether pioglitazone alone has effects on Tlr4, Myd-88 and Nf-kb1 expression in mice tumor tissue. As shown in figure 2A, Tlr4 expression was significantly decreased by 10 (P<0.05), 50 and 100 mg/kg/day (P<0.01) doses of pioglitazone. Myd-88 expression at all of the doses (P<0.01) and the expression of Nf-kb was reduced significantly at doses of 10 (P<0.05), 50 (P<0.01) and 100 mg/kg/day (P<0.01). In the other groups that were injected with LPS treated cells, pioglitazone injection significantly reduced the expression of Tlr4, Myd-88 and Nf-kb in all doses in comparing with their control group (Figure 2B).

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**Figure 1.** Mean melanoma tumors size in different groups. Mice were injected with LPS treated or untreated cells. Tumor bearing animals were treated for 10 days with different concentration of pioglitazone. *P<0.05, **P<0.01, in comparison with control group and #P<0.01 compare LPS group. Each graph has been represented as Mean ± SEM.
Figure 2. Effect of LPS and pioglitazone on the gene expression of Tlr4, Myd-88, and Nf-kb in melanoma tumors. A) Experimental groups were injected by B16F10 cells and then administered pioglitazone as described in the materials and methods section. B) Mice were injected with LPS treated B16F10 cells. Following the development of tumors mice were injected with different concentration of pioglitazone. The mRNA level of Tlr4, Myd-88, and Nf-kb was determined by QRT-PCR. Fold changes relative to control are presented as Mean±SEM. *P<0.05, ** P<0.01, and *** P<0.001 compared with control group.

Pioglitazone decreases TLR4 protein expression in mice melanoma

All the samples, including control, LPS and pioglitazone 50 and 100 (mg/kg/day), groups with or without LPS, were positive for TLR-4 protein expression. As shown in figure 3 compared with the control group, the TLR-4 expression was significantly increased in the LPS group (P<0.001). We found reduced TLR-4 protein expression in the pioglitazone group, but it was not significant when compared with the control group (P<0.05). Interestingly, we found reduced TLR-4 protein expression in the group that received LPS and pioglitazone 50 and 100 (mg/kg/day) compared with the LPS group (P<0.001).

Pioglitazone inhibits TNF-α production in mice tumor lysate

To examine the effect of LPS on TNF-α concentration in serum and tumor lysate its level was determined by Elisa kit. Our results have shown that the mice group that was injected with LPS treated cells, the amounts of TNF-α in tumor tissue was significantly higher than the control group (Figure 4A).

The results illustrated that pioglitazone in each mice with or without LPS–treated cells had no effect on TNF-α concentration in mice serum compared to control group (Figure 4B).

DISCUSSION

The present study provided evidence that pioglitazone reduced melanoma progression by suppressing the TLR4 signaling pathway and inflammatory cytokine.

Various studies strongly suggest that PPARγ agonists in addition to the anti-diabetic effect, can induce cell growth arrest, apoptosis, and terminal differentiation in many human malignant tumors (28).

PPAR family plays a regulatory role in cell cycle and growth (29). Activation of PPAR agonists can lead to inhibition of tumorigenesis and provide new insights to cancer therapy (28,30,31).

Rosiglitazone, a PPAR γ agonist was shown to inhibit hepatocellular carcinoma (32), and gastric cancer cell growth (33). Also, troglitazone the other PPAR γ agonist has the same effect on prostate cancer cells (34). PPAR agonists exert this regulatory effect via regulating the expression and blocking the oncogenic proteins (20). In our study, we found that PPARγ agonist...
significantly reduced tumor size and melanoma cell proliferation.

Activation of TLR4 by LPS significantly enhanced the survival of melanoma cells while TLR4 inhibition led to the death of melanoma cells (35). In the present study, we also observed that LPS increased expression levels of Tlr4, Myd-88, and Nf-κb1, whereas pioglitazone reversed this effect. So we conclude that PPARγ agonist inhibited the progression of melanoma cells via blocking the TLR4 pathway.

In general, our result suggested that activation of PPARγ suppressed proliferation and induced apoptosis of melanoma cells via inhibiting TLR4-dependent NF-κb pathway.

Several studies are warranted to investigate PPARγ expression in skin cancer and the mechanisms by which this receptor affect skin carcinogenesis, such as, proliferation, apoptosis, inflammation and angiogenesis (16,36). The anti-proliferative activity of PPARγ agonists opens up interesting new perspectives for melanoma treatment. Freudlsperger et al. have been shown a significant inhibitory effect of glitazones on the proliferation of human melanoma cells in vitro (37).

**Figure 3.** Immunohistochemical evaluation of TLR4. A) TLR-4, IHC quantifications relative to respective negative control in melanoma tumors. B) Pictures are representative fields of tumor staining for each tumor [original magnification, ×400]. ***P < .001, in comparison with control group, ####P < .001, compare LPS group.
Figure 4. Effect of pioglitazone on TNF-α production in mice serum and tumor tissue lysate. *P<0.05, in comparison with control group and #P<0.05 compare LPS group. Each graph has been represented as Mean ± SEM.

For the first time, in the present study, we show that pioglitazone can significantly decrease Tlr4, Myd-88 and Nf-κb1 gene expression in vivo with or without LPS stimulation. Also treatment of mice with pioglitazone resulted in a significant reduction in the TLR4 protein expression in tumor lysate in a group that received LPS stimulated cells. It has been known that TLR4 expression in many tumors or cell lines was up-regulated (38). Previous studies demonstrated that pioglitazone attenuated AngiotensinII-induced inflammatory response in cardiac fibroblast cells through inhibition of the TLR4 signaling pathway (39) and the other PPARγ agonist, roziglirazone suppressed the expression of Tlr4 mRNA and protein in alveolar macrophages (40), which are consistent with our study. A recent study highlighted that LPS increased expression levels of Tlr4, Myd-88, whereas PPARγ agonist, roziglirazone resisted this effect in esophageal cancer cells(20). It has been shown that PPARγ 15d-PGJ2 regulates Tlr4 mRNA and protein expression in HT-29 cells(41). Also, it has been shown that Hypaphorine, an indole alkaloid from Erythrina velutina, protects HMEC-1 cells against LPS-induced inflammation by inhibiting PI3K/Akt/mTOR signaling pathways, followed by the interactive modulation of TLR4 with PPAR-γ(42). 6-Shogaol, a pungent agent isolated from Zingiber officinale Roscoe, has been known to have anti-tumor and anti-inflammatory effects.It has been shown that 6-Shogaol could increase the expression of PPAR-γ. It can inhibit LPS-induced inflammation BV2 microglia cells by activating PPAR-γ(43). Similarly activation of PPAR-γ by nuciferine, a bioactive component extracted from the lotus leaf, can inhibit the LPS-induced inflammation in BV2 cells(44).

In the present study, we also observed that LPS increased the amount of TNF-α in tumor lysate but not in mice serum and pioglitazone can inhibit LPS effect on TNF-α production. It has been shown the role of PPARγ in the regulation of NF-kB signaling by modulating Tlr4 expression and TNF-α production in LPS stimulated monocyte Leukemia cells (45).

The other PPARγ agonists, Glitazones, have demonstrated the capacity to diminish inflammatory cytokine production, most notably, that of tumor necrosis factor-α. Several lines of evidence indicate significant thiazolidinedione mediated antitumor activity.

These results indicated that pioglitazone may counteract LPS-stimulated inflammation via the blockade of TLR4. Perviously in our in vitro study, we evaluated the effects of TLR4 inhibitor in combination with pioglitazone on the production of TNF-α in B16F10 cells treated with LPS or nothing. We observed that pioglitazone and TLR4 inhibitor, CLI-095, co-treatment synergistically inhibited LPS-induced production of TNF-α. This indicates the inhibitory effect of pioglitazone on the TLR4 signaling. Therefore, in terms of in vivo conditions, pioglitazone has the same effect on this pathway.

In summary, these findings provide the evidence for beneficial effects of PPARγ activator pioglitazone to counter-regulate melanoma cancer by affecting on TLR4 signaling pathway. More importantly, the anti-inflammatory action of
pioglitazone via interfering with the TLR4-dependent signaling pathway (TLR4/MyD-88/NF-kB) work against melanoma.

CONFLICT OF INTEREST

None.

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