Bacillus Calmette-Guérin induces the expression of peroxisome proliferator-activated receptor gamma in bladder cancer cells

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Abstract. Bacillus Calmette-Guérin (BCG) is considered to be one of the most effective treatments for superficial and *in situ* bladder cancer. The exact mechanism of the antitumor activity of BCG is not completely understood. Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors that is involved in cell growth and differentiation as well as inflammatory processes. PPARγ is expressed in normal urothelium and a lack of expression was associated with bladder cancer progression. We analyzed whether PPARγ is involved in the inhibition of bladder cancer cell survival by BCG. PPARγ expression in murine MB49 and human T24 bladder cancer cells was evaluated employing immunofluorescence and inmunohistochemistry techniques. *In vitro* cell viability and nitric oxide (NO) production was evaluated by using MTS and Griess reagent respectively. Our results show that BCG induced the cytoplasmatic expression of PPARγ in bladder tumor cells *in vitro* and *in vivo*. BADGE, antagonist of this receptor, abrogated *in vitro* BCG-mediated cell cytotoxicity. Natural agonist 15-deoxy-Δ12,14 prostaglandin J2 (15-d-PGJ2) but not rosiglitazona (RO), a synthetic agonist, induced *in vitro* inhibition of cell viability of both cancer cell lines and the effect was partially reversed by BADGE. We also determined whether the activation of PPARγ could inhibit NO production, which is considered a survival factor for bladder tumor cells. Both 15-d-PGJ2 and RO significantly inhibited the NO production in T24 and MB49 cells by PPARγ-independent pathway since it was not antagonized by BADGE.

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Thus, our results show that BCG induces functional PPARγ in bladder tumor cells *in vivo* and *in vitro*, being these receptors intrinsically involved in the antitumor activity of BCG.

Introduction

Intravesical administration of Bacillus Calmette-Guérin (BCG) plays a major role in the treatment and prophylaxis of recurrent superficial and *in situ* bladder carcinoma (1).

The exact mechanism of the antitumor activity of BCG is not completely understood but it seems to involve direct effects on tumor cells and others mediated by immune cells. It is well known that BCG evokes an early local and non-specific immune cell reaction including increase in cytokine production and finally Th1 cell-regulated cytotoxicity (2,3). Previously, we have demonstrated that BCG induced *in vitro* bladder tumor cell death by a mechanism independent of concomitant production of nitric oxide (4).

PPARγ is a member of the nuclear receptor superfamily of ligand-activated transcription factors and functions as a regulator of adipocyte differentiation and lipid metabolism (5). PPARγ is expressed at high levels in white adipose tissue and is also highly expressed in many other specialized cells, such as macrophages (6), T cells (7), smooth muscle cells of the arterial wall (8) and epithelial cells, including rodent and human urothelial cells (9,10). Receptor activation has also been implicated in inflammation and tumorigenesis. PPARγ is up-regulated in some malignant tissue, and its ligands induce terminal differentiation and/or growth inhibition of human breast, colon, prostate, gastric and lung cancer cells (11,12). PPARγ can be activated by a number of ligands, either natural such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-PGJ2) (13) or synthetic such as antidiabetic thiazolidinediones (TZD) (14). Activation of PPARγ by troglitazone, a TZD, induces differentiation and reversal of malignancy in different tumors (15,16).

The function of PPARγ on urothelium is unknown and the finding that it is expressed in urothelial tumor cells raised questions about the role of PPARγ and its ligands in bladder cancer. It has been described that activation of PPARγ in bladder cancer cell lines *in vitro* induced growth inhibition and even cell death (10,17). Although there is a discrepancy

about the significance of PPARγ expression in bladder tumors (10,18,19), the consensus is that the presence of PPARγ in bladder tumor cells should be considered an indicator of good prognosis or, conversely, the lack of expression of PPARγ can be associated with tumor progression. Thus, one might assume that reexpression induction of PPARγ could induce regression of bladder tumors.

In order to investigate whether PPARγ are linked to BCG-induced bladder tumor cell death, we first determined the PPARγ expression in tumor cells followed by an evaluation of their participation in BCG-mediated tumor cell cytotoxicity. Our results demonstrate that BCG induced PPARγ expression in bladder tumor cells *in vitro* and *in vivo*. Furthermore, a PPARγ antagonist abrogated *in vitro* BCG-mediated cell cytotoxicity. In summary, we can suggest that an efficient BCG therapy involves induction of PPARγ in bladder cancer cells.

Materials and methods

Tumor cell lines. Human T24 (obtained from ATCC, Bethesda, MD) and murine MB49 (obtained from Dr E.C. Lattime, Thomas Jefferson University, Philadelphia, PA) bladder cancer cell lines were cultured in MEM (41500, Gibco BRL) supplemented with 3 mM L-glutamine, 80 μg/ml gentamycin and 8% fetal calf serum (FCS).

BCG. Living organisms of an attenuated strain of Myco-bacterium bovis [Pasteur 1172 P2 strain, containing 3×10^6 colony forming units (CFU) per mg] were obtained from Instituto Nacional de Producción de Biológicos ANLIS C.G. Malbrán, Buenos Aires, Argentina.

Immunofluorescence of PPARγ in bladder cancer cells. T24 or MB49 cells (2×10^5/wells) were cultured for 48 h on cover slides in 6-well culture dishes, with or without 2×10^6 CFU of BCG/ml. Slides were then gently washed with cool saline buffer and processed for immunofluorescence. Briefly, slides were first permeabilized and unspecific blocking was performed using Tween-20 1% plus casein 10% in PBS for 90 min at room temperature. Rabbit polyclonal antibody anti-PPARγ (H-100, Santa Cruz Biotechnology, Inc) diluted 1:50 was used overnight. Goat anti-rabbit IgG-fluorescein conjugated, diluted 1:50, was used as secondary antibody (Chemicon International, Inc) for 60 min and counterstained with ethidium bromide. Slides were observed using a fluorescence Nikon Eclipse E400 microscope and photographs were obtained using a digital camera, Nikon Cool PIX 995, at a magnification x200.

In vivo treatment of MB49 tumor bearing mice. To assay if BCG was able to induce PPARγ expression *in vivo*, MB49 cells (4×10^5/0.1 ml) were injected subcutaneously into the flanks of syngeneic C57BL/6J mice as previously described (4). BCG (2×10^6 CFU/ml) was injected intratumorally once a week starting 24 h after tumor cell inoculation. After 14 days, mice were necropsied and tumors were fixed in 10% neutral buffer formalin and processed for routine hematoxilin-eosin and immunohistochemistry.

Mice (approximately 8 weeks old) were obtained from our Animal Care Division. Animal care was provided in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals (NIH publications 1986).

Inmunohistochemistry. Paraffin-embedded sections from MB49 bladder tumors (n=6; 3 controls and 3 from BCG-treated mice), were used to analyze the PPARγ expression. Inmunohisto-chemical staining was performed using an avidin-biotinyl peroxidase complex as previously described (20) with the unique modification that the first antibody was PPARγ specific (H-100, Santa Cruz Biotechnology, Inc).

Assay of in vitro tumor cell survival and nitric oxide production. Tumor cell lines (1×10^4 cells/200 μl) were cultured in 96-well plates (Greiner Labortechnik GmbH, Frickenhausen, Germany) at 37°C in a 5% CO_2 humidified atmosphere. After 24 h incubation, different concentrations (0.1, 1, 10 and 50 μM) of natural (15-d-PGJ2; Cayman Chemical, USA) or synthetic (rosiglitazona, RO; ELEA, Buenos Aires, Argentina) PPARγ agonists with or without BCG (2×10^6 CFU/ml) were added. The PPARγ antagonist, bisphenol A diglycidyl ether (BADGE; FLUKA Chemical GmbH, Buchs, Switzerland), was also used. Cells were cultured for an additional 48 h. The cytostatic/cytotoxic effect of BCG was assessed using a non-radioactive cell titer (Cell Titer 96 Aqueous, MTS; Promega). Nitric oxide (NO) production in tumor cell supernatants was determined by using Griess reagent (21). Different concentrations of sodium nitrite were used to construct a standard curve. Results of NO production are expressed as nitrite μM. Absorbance was measured at 492 or 550 nm for MTS or NO determination, respectively, using an ELISA reader (Multiskan Ascent, Labsystem).

Statistical analysis. Results are expressed as mean ± SD of almost six replicates per group. Significance of differences was calculated by one-way ANOVA, by using Graph Pad InStat statistical package (version 3.01). Bonferroni contrast was used to compare the *in vitro* assays. All experiments were repeated at least 3 times with similar results.

Results

BCG induced PPARγ expression in bladder cancer cells in vitro and in vivo. As PPARγ has been associated with the inhibition of tumor cell growth, we studied whether BCG activity on bladder cancer cells was associated with the induction of PPARγ expression. Neither MB49 nor T24 cell lines expressed PPARγ in basal conditions as determined by immunofluorescence. However, the *in vitro* addition of BCG to cultured cells induced cytoplasmatic PPARγ expression in both cell lines (Fig. 1A-D). BADGE abrogated *in vitro* BCG-mediated cell cytotoxicity (Fig. 2) suggesting that this receptors are involved death of tumor cells.

Immunohistochemical studies showed that in MB49 tumors, growing in the flank of C57Bl/J6 mice. BCG treatment also induced PPARγ expression on tumor cells, while control tumors were negative (Fig. 1F and E respectively).

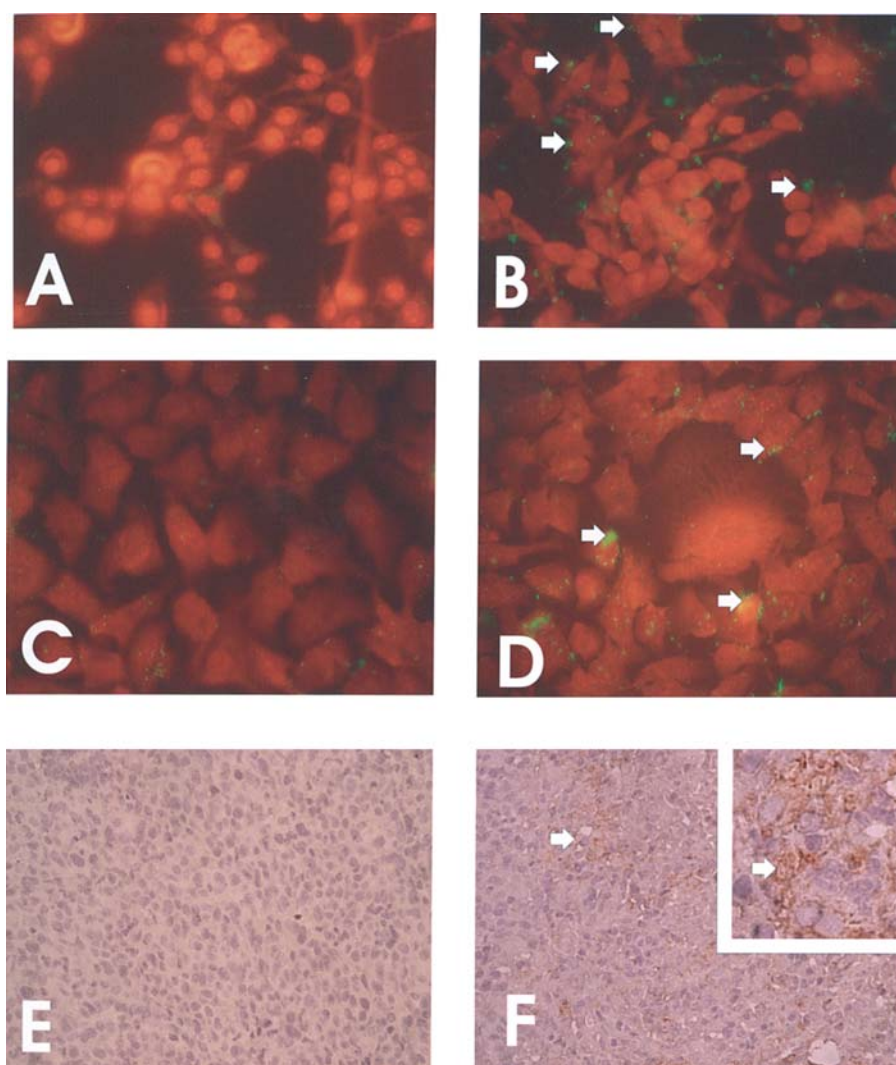*Effect of PPARγ agonist on tumor cell survival and nitric oxide production*. 15-d-PGJ2, the major natural agonist of

Figure 1. Representative immunostaining for PPARγ in bladder cancer cell lines *in vitro* and *in vivo*. Negligible PPARγ expression was found in MB49 (A) and T24 (C) cells *in vitro* and in MB49 cells growing s.c. in C57BL/J6 mice (E). Upon BCG treatment, immunoreactive PPARγ was detected either *in vitro* in MB49 cells (B) and T24 cells (D) or *in vivo* (F). Photograph magnification, x100; insert, x400.

PPARγ, induced *in vitro* inhibition of cell viability of both murine and human bladder cancer cell lines, with human T24 being more sensitive (40% inhibition at 0.1 μM 15-d-PGJ2 and 60% at 10 μM) than murine MB49 cells (approximately 20% inhibition at all concentrations) (Fig. 3). It is noteworthy that the 15-d-PGJ2 inhibitory effect was only detected when cells were cocultured in the presence but not in the absence of BCG. We also demonstrated that the inhibiton of cell viability by 15-d-PGJ2 was PPARγ-dependent since it was partially reversed by BADGE (40 μM). By the contrary, RO, alone or in combination with BADGE, did not affect the cell viability of either cell line (Fig. 3).

As PPARγ are negative regulators of inflammation and, thus, of NO production, and as we have previously suggested that NO could be a survival factor for bladder tumor cells (4), we evaluated whether 15-d-PGJ2 and RO could inhibit NO production upon BCG treatment. Our results show that both ligands significantly inhibited NO production in T24 and MB49 cells. In T24 cells, the inhibition by RO was dose-dependent (10% inhibition at 0.1 μM and 28% at 1 μM, reaching a plateau of 35% inhibition at 10 μM). The inhibitory effect of 15-d-PGJ2 was less evident (approximately 25% at
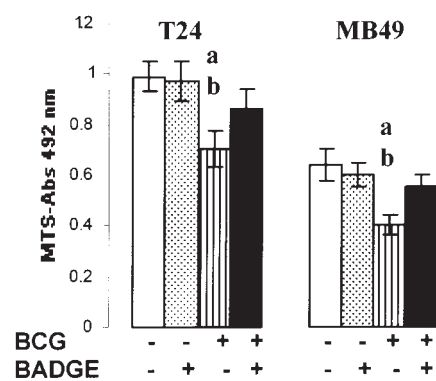

Figure 2. Subconfluent monolayers of bladder cancer cell lines growing in 96-well plates were incubated for 48 h with or without viable BCG, with or without BADGE (40 mM). Cell viability was evaluated by MTS, and referred to as fold of control. a, p<0.001 between cells with or without BCG; and b, p<0.01 between cells with BCG and BCG + BADGE.

all doses). In this case, the effect of both ligands on NO production was PPARγ-independent since it was not antagonized by BADGE. MB49 cells were more sensitive to NO
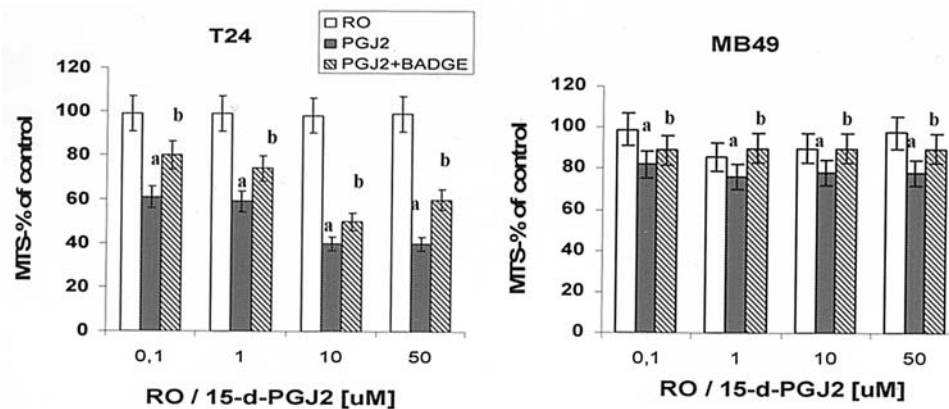
Figure 3. Subconfluent monolayers of bladder cancer cell lines growing in 96-well plates were incubated for 48 h with BCG plus RO or 15-d-PGJ2, with or without BADGE (40 mM). Cell viability was evaluated by MTS, and referred to as fold of control. 15-d-PGJ2 significantly inhibited the cell viability (a, $p < 0.001$) and this effect was reversed by BADGE (b, $p < 0.01$).
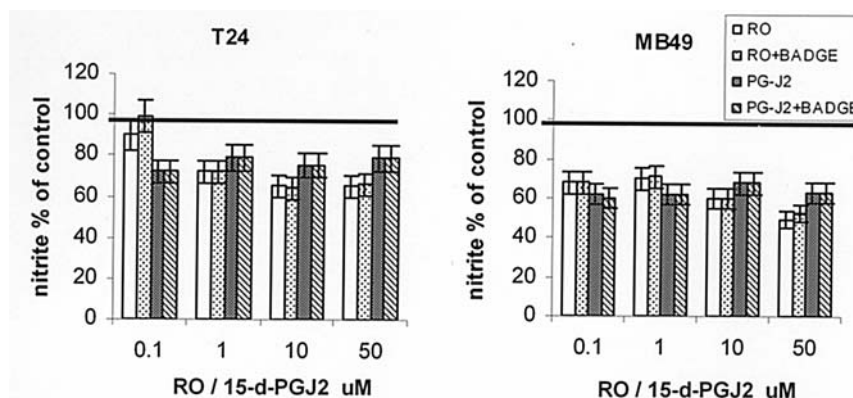

Figure 4. NO production by tumor cell lines was evaluated in the supernatants by using Griess reagent. Both RO and 15-d-PGJ2 at all concentrations inhibited the NO production induced by BCG ($p < 0.01$) and this was not reversed by BADGE.

inhibition by RO and PGJ2 (30-40% inhibition) than T24 cells (Fig. 4). Our results suggest that inhibition of NO upon BCG treatment appears to be PPARγ-independent.

Discussion

Intravesical administration of BCG is used for therapy and prophylaxis of recurrent superficial and *in situ* bladder carcinoma (1). The mode of action against bladder carcinoma remains unclear, but it is well known that BCG evokes an early local and non-specific immune cell reaction (2,3). A direct effect on tumor cells was also observed although this pathway has been less explored. Results from our laboratory have shown that BCG induced *in vitro* an NO-independent inhibition of bladder cancer cell growth. Furthermore, the *in vivo* inhibition of NO improved BCG therapy (4).

The present experiments were designed to better understand the mechanisms exerted by BCG on bladder tumor cells.

Several reports have demonstrated that PPARγ are involved in cancer cell growth inhibition (10-12,15,16). It was described that transitional bladder epithelia expresses PPARγ and its lack of expression in bladder cancer is associated with a bad prognosis (10,18). Thus, we studied whether PPARγ are involved in BCG-induced bladder tumor cell death. We observed that PPARγ expression was weak or absent in MB49

and T24 cells and that the addition of BCG induced its cytoplasmatic expression (Fig. 1). When different PPARγ agonists were used *in vitro* inhibition of cell viability was only detected with 15-d-PGJ2 and not with RO. The tumor cell lines were not equally sensitive to 15-d-PGJ2, murine being more resistant than human cells. It is noteworthy that the effect of 15-d-PGJ2 was only detected in the presence of BCG. According to these results, we can assume that BCG has the ability to induce functional PPARγ which can respond only to the endogenous ligand.

PPARγ ligands can also exert a PPARγ-independent effect (16). In our system, we demonstrated that inhibition of cell viability by 15-d-PGJ2 was PPARγ-dependent since BADGE, a specific PPARγ antagonist, reversed the prostanoid effect (Fig. 3). BADGE also directly reversed BCG-mediated cell death (Fig. 2), confirming that PPARγ are involved in BCG cytotoxic activity. Thus, we can assume that functional PPARγ induced by BCG are involved in any of the pathways that lead to tumor death. It can be hypothesized that the endogenous concentration of 15-d-PGJ2 after BCG therapy could provide a better response to this biotherapy.

Since it is known that PPARγ down-regulate iNOS expression and function (22) and BCG response is associated with NO production, we assayed whether 15-d-PGJ2 and RO modulated the NO production concomitantly with BCG.

A combined treatment of BCG with either RO or 15-d-PGJ2 inhibited the NO production. This inhibitory activity was PPARγ-independent since it was not reversed by BADGE. No modifications of NO levels were detected in the absence of BCG. Although a PPARγ-independent mechanism has been previously described for synthetic agonist concentrations of >10 μM (23), we detected the inhibition of NO production from 0.1 μM of RO or 15-d-PGJ2.

It is of interest that, in bladder cancer cells, PPARγ agonists may function via either PPAR-dependent or -independent pathways, being cell death-dependent, while the inhibition of NO production seems to act via a PPARγ-independent pathway.

Our previous results indicate that expression of inducible NO synthase (iNOS) is a bad prognosis factor for bladder cancer patients (20). Thus, we can hypothesize that RO or 15-d-PGJ2, by inhibiting NO production, might be a valuable therapeutic tool for patients with tumors expressing iNOS. However, this approach needs further research as both TZD and 15-d-PGJ2 are immune suppressors (7,22,24) and their use may abrogate the immune response to BCG.

In summary, our results show that BCG induces functional PPARγ in bladder tumor cells *in vivo* and *in vitro* and that these receptors are intrinsically involved in the antitumor activity of BCG. Levels of endogenous agonists, such as 15-d-PGJ2, could predict whether BCG immunotherapy would be successful or not.

Acknowledgements

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