Rosiglitazone impairs proliferation of human adrenocortical cancer: preclinical study in a xenograft mouse model

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Abstract

Adrenocortical carcinoma (ACC) is a rare aggressive tumor with a poor prognosis. The lack of a specific and effective medical treatment is due to the poor knowledge of the mechanisms underlying tumor growth. Research on potential drugs able to specifically interfere with tumor proliferation is essential to develop more efficacious therapies. We evaluated for the first time the \textit{in vivo} effect of rosiglitazone (RGZ), an anti-diabetic drug with \textit{in vitro} anti-tumor properties, on ACC proliferation in a xenograft model obtained by s.c. injection of human ACC H295R cells in athymic mice. When the tumor size reached 5 mm, animals were allocated to 5 mg/kg RGZ- or water-treated groups. Tumor volume was measured twice a week. A significant reduction of tumor growth in RGZ versus control (control) group was observed and was already maximal following 17 day treatment (1 \textit{T/C} = 75.4\% (43.7–93.8\%)). After 31 days of treatment, mice were killed and tumor analyzed. Tumor histological evaluation revealed characteristics of invasiveness, richness in small vessels and mitotic figures in control group, while RGZ group tumors presented non infiltrating borders, few vessels, and many apoptotic bodies. Tumor immunohistochemistry showed that Ki-67 was reduced in RGZ versus control group. Quantitative real-time RT-PCR demonstrated a significant reduction in the expression of angiogenic (VEGF), vascular (CD31), proliferation (BMI-1), and anti-apoptotic (Bcl-2) genes in RGZ versus control group tumors. The same inhibitory effects were confirmed in \textit{in vitro} RGZ-treated H295R. Our findings support and expand the role of RGZ in controlling ACC proliferation and angiogenesis \textit{in vivo} and \textit{in vitro}.

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Introduction

Adrenocortical carcinoma (ACC) is a rare (1:4×10\textsuperscript{6} annual estimated incidence) and aggressive endocrine tumor with a poor prognosis, generally characterized by a limited response to radio/chemotherapy (Allolio \& Fassnacht 2006). At present, early diagnosis followed by total surgical tumor resection is the only valuable option for ACC cure. Prognosis depends on the tumor stage at surgery – mean survival rate at 5 years is 16–38\% (Allolio \& Fassnacht 2006); however, metastatic disease reduces survival to <10\% (Fassnacht \textit{et al.} 2009). The lack of a specific and effective medical regimen is due to the poor knowledge of the mechanisms underlying malignant tumor transformation and progression. Mitotane is the only pharmacological adjuvant treatment available for ACC in advanced disease (Luton \textit{et al.} 1990, Terzolo \textit{et al.} 2007), and its combination with other chemotherapeutic drugs has led to variable results (Khan \textit{et al.} 2000, Berruti \textit{et al.} 2005). However, its effects are mainly due to its adrenocortical cytotoxicity. Thus, the development of new efficacious and less toxic drugs, specific for ACC, to be eventually combined with mitotane (Barlaskar \textit{et al.} 2009) are required.

The peroxisome proliferator-activated receptor (PPAR)-\gamma is a ligand-activated transcription factor,
its thiazolidinedione (TZD) ligands, used in type 2 diabetes therapy, have been shown to exert additional anticancer effects in several solid tumors (Panigrahy et al. 2005). Although the antitumor action of TZDs (due to their anti-proliferative, anti-angiogenic, and anti-inflammatory effects) have been well documented in in vitro and animal studies, the results which have emerged from the few clinical trials are still quite controversial (Burton et al. 2008). Very few studies have been conducted on TZD effects in adrenal cancer. In particular, rosiglitazone (RGZ), the TZD with the highest affinity for the receptor, has been demonstrated to interfere with cancer growth by blocking cell proliferation/migration and to induce cell differentiation/apoptosis in the ACC cell line H295R (Betz et al. 2005, Ferruzzi et al. 2005, Cantini et al. 2008).

In this study, we investigated the effects of RGZ in a human ACC xenograft model as obtained by s.c. H295R injection into athymic mice. In particular, we evaluated RGZ inhibition on tumor growth in vivo and evidenced some specific target genes involved in the interfering effects of RGZ both in vivo and in vitro.

Materials and methods

Materials

Media and sera for cell cultures were from Lonza (Milan, Italy) and tissue plasticware from Corning (Milan, Italy). Other reagents were obtained from Sigma–Aldrich, except where differently indicated. RGZ was obtained from Alexis Biochemicals (San Diego, CA, USA).

Cell cultures

The human ACC cell line H295R was obtained from the American Type Culture Collection (Manassas, VA, USA). H295R cells were cultured in 10% fetal bovine serum (FBS) (Euroclone, Celbio, Milan, Italy), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, DMEM/F-12 medium enriched with a mixture of insulin/transferrin/selenium (Sigma–Aldrich) at 37 °C in a humidified 5% CO2 atmosphere. Subconfluent cells were treated for 24 h with 20 μM RGZ, following overnight starvation.

Xenograft model

A total of 33 female athymic CD1 nude mice (6–8-week-old, Charles River Laboratories, Italy) were inoculated subcutaneously with H295R cell suspension (7×10⁶ cells/100 μl) in two independently performed experiments. Data obtained in the two experiments were pooled. Subcutaneous tumor growth was daily monitored, and when solid tumors reached a 5 mm mean diameter (9 days after H295R inoculation), the animals were assigned to be orally treated with RGZ (5 mg/kg in 100 μl, 6 days/week) or vehicle, according with randomization decided before s.c. injection of H295R. Tumor volume (mm³) was the mean of values estimated by two independent investigators from two dimensional tumor measurements by the formula: length×width²/2. After 31 days of oral treatment (in accordance with RGZ treatment interval of 4 week already described in literature (Heaney et al. 2002, Dai et al. 2008)), mice were killed by cervical dislocation, and tumors were collected and split for mRNA or histological/immunohistochemical analyses.

Drug anti-tumor activity was evaluated as the tumor growth inhibition percentage calculated by the 1 − treated/control tumor volume ratios (1−T/C) (Teicher 1998, Johnson et al. 2001, Hollingshead 2008). Data were expressed as the median with the minimum–maximum value interval.

Drug tolerability was assessed in tumor-bearing mice in terms of: a) lethal toxicity, i.e. any death in treated mice occurring before any death in control mice; b) body weight loss percentage =100−(body weight on day x/body weight on day 1×100), where x represents a day after or during the treatment period (Teicher 1998, Johnson et al. 2001, Hollingshead 2008).

Animal studies were performed in compliance with an institutionally approved protocol and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tumor measurements, as well as tumor histologic/immunohistochemical and gene expression analyses, were performed by independent investigators working in blind conditions.

Histologic and immunohistochemical examination of xenografts

Tissues were fixed in 10% buffered formalin and paraffin-embedded. Five-micrometer sections were hematoxylin and eosin (H/E) stained for histologic evaluation, or used for immunohistochemistry. Apoptotic body (AB) and mitotic (MI) indices were obtained by counting 1000 cells in H/E-stained sections. Apoptotic bodies were recognized as shrunk cells with compact or segregated, sharply delineated masses of chromatin in nodular, ring-like or bead-like patterns. If present, the cytoplasm was deeply eosinophilic. As a result of cellular shrinkage, apoptotic cells were frequently surrounded by a clear halo. Apoptotic bodies also consisted of dense
extracellular or intracellular chromatin fragments, with or without associated cytoplasm. Mitotic figures were identified by morphologic features of metaphase, anaphase, or telophase.

Immunohistochemical analysis with mouse anti-human Ki-67 monoclonal MIB1 antibody (Dako, Glostrup, Denmark) was performed with the Ventana Benchmark XT system (Ventana Medical Systems, Tucson, AZ, USA). Nuclei were hematoxylin-counterstained. Ki-67 positive nuclei were counted on 1000 tumor cells. Negative controls were performed by omitting the primary antibody.

**RNA isolation and quantitative real time RT-PCR**

Xenograft tumors, snap frozen in liquid nitrogen, and pelleted H295R cells were processed for RNA extraction. Total mRNA isolation and quantitative real time RT-PCR were performed as detailed elsewhere (Lombardi et al. 2008) using specific primers/probes (Assay on Demand; Applied Biosystems, Warrington, UK). The amount of target mRNA was given by $2^{-\Delta\Delta C_t}$ calculation, following normalization to an endogenous reference gene (18S, conserved in the mouse and humans, for xenograft tumor analysis; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for H295R analysis) and relative to a calibrator (Stratagene, La Jolla, CA, USA).

**Statistical analysis**

The statistical analysis was performed using SPSS 12.0 (SPSS 12.0; SPSS Inc). One-way ANOVA followed by Dunnett’s post hoc test was applied for multiple comparisons, whereas Student’s t-test was used for comparisons of two classes of data. A $P$ value <0.05 was considered significant.

**Results**

**RGZ treatment interferes with tumor growth in the xenograft model of ACC**

All H295R injected mice ($n=33$ from two independent experiments) developed a detectable tumor (100% overall tumor take rate), confirming the aggressiveness of ACC-derived cells. Tumors for each group of treatment, control and RGZ, were followed and measured during the 31 days of treatment. Tumor volume was calculated for each mouse at each time point. Figure 1 shows the tumor growth rate in the two treatment groups. Data were plotted as mean tumor volume (Fig. 1) in each group over time. RGZ administration resulted in a reduced increase in tumor volume, which was already statistically significant after 3 days of treatment (Fig. 1). After 3 days of treatment, RGZ inhibitory effect was already relevant ($1-T/C=45.3\% \ (15.5-74.4\%)$), reached a maximum after 17 days ($1-T/C=75.4\% \ (43.7-93.8\%)$) and remained constant up to the end of the treatment ($1-T/C=74.5\% \ (25.3-94.1\%)$). Interestingly, the tumor volume increased compared to the initial volume at any time point in the control animals, and such an increase became statistically significant starting from day 17. Conversely, in the RGZ-treated mice, there was an initial reduction in the tumor size, followed by a slow increase in the long term, which was statistically significant only from day 27 (Fig. 1). Moreover, the differences in the tumor volume increase versus day 0 were statistically significant at any time point between control- and RGZ-treated groups. The drug was well tolerated without lethal toxicity and body weight loss during treatment (data not shown).

The xenograft tumors obtained by s.c. injection of H295R (Fig. 2) were histologically similar to the adrenocortical tumor from which H295R cells were derived confirming previous observations (Logić et al. 2000).

In the control group, the tumors displayed a solid, diffuse architecture (Fig. 2A) and consisted of rather small, uniform cells with coarse chromatin and prominent nucleoli. A mixture of large caliber vessels and a disorganized network of small-caliber vessels were seen (Fig. 2C). A remarkable number of mitotic
figures were present, often with atypical forms (Fig. 2E). Conversely, in the RGZ group, the tumors showed well-demarcated borders (Fig. 2B), a lower degree of vascularization (Fig. 2D), a greater number of apoptotic bodies and a lower MI (Fig. 2F). Table 1 shows the mean percentage of MI and ABI detected in H/E-stained tumor sections in control and RGZ groups. RGZ-treated group showed a statistically significant decrease in the percentage of mitosis \((P < 0.05, n=10\) animals for each group of treatment obtained in two independent experiments) and a statistically significant increase in the percentage of apoptotic figures \((P < 0.05, n=10\) animals for each group of treatment obtained in two independent experiments) as compared to control group.

Immunohistochemical analysis of tumor sections from xenografts revealed that RGZ treatment was associated with a reduction in Ki-67 staining in cell nuclei (Fig. 3B) as compared with control (Fig. 3A), suggesting an inhibitory effect of RGZ on tumor proliferation. RGZ-treated group showed a statistically significant decrease in the percentage of Ki-67 labeling index as compared with control group \((P < 0.001, n=10\) animals for each group of treatment obtained in two independent experiments), Table 1.

Molecular analysis of the xenograft tumors was performed on total mRNA extracted from control and RGZ-treated animals. Similarly to data obtained in H295R cells, PPAR\(\gamma\) was expressed in all xenografts and not affected by RGZ treatment, as detected by quantitative real-time RT-PCR (PPAR\(\gamma\) versus 18S expression \(\pm\) s.e.m. in control: 3.4 \(\pm\) 1.8 and RGZ: 3.1 \(\pm\) 1.3 groups, \(n=8\)). Tumor cell proliferation was reduced by 31 days RGZ treatment as demonstrated by the statistically significant decrease in the proliferation marker.
BMI-1 (Fig. 4A). RGZ not only affected tumor proliferation but also the angiogenic process supporting tumorigenesis. In fact, both the angiogenic factor VEGF and the endothelial marker CD31 were highly expressed in the untreated control tumors, while their expression was significantly reduced in tumors from RGZ-treated group (Fig. 4A). Confirming the histological features, TaqMan analysis demonstrated a significant reduction in the expression of the anti-apoptotic marker Bcl-2 in the RGZ-treated versus the untreated control group (Fig. 4A). Finally, in tumors from both groups we measured expression of seladin-1/DHCR24, the gene encoding 3β-hydroxysterol Δ-24-reductase (DHCR24), which converts desmossterol into cholesterol (Greeve et al. 2000). In fact, seladin-1 mRNA levels have been demonstrated to be significantly lower in adrenocortical cancer and in the derived primary cell cultures compared to adenoma and normal adrenal and their derived primary cell cultures (Luciani et al. 2004). At variance with the decrease found in all the above described genes, seladin-1 mRNA levels were slightly, although not significantly, up-regulated in the RGZ-treated group (Fig. 4A).

RGZ effects on expression of adrenal tumorigenesis genes in in vitro H295R cultures confirm results obtained in the mouse xenograft model

To confirm that the effects observed on tumor growth following mouse treatment with RGZ were also due to the direct effect exerted by RGZ on H295R and not only on the mouse tissue tumor micro-environment, we treated H295R with RGZ in vitro and analyzed the expression of the adrenal tumorigenesis genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>RGZ</th>
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<tbody>
<tr>
<td>VEGF</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>CD31</td>
<td>66.4</td>
<td>50.3</td>
</tr>
<tr>
<td>BMI-1</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>4.1</td>
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*P<0.05 and †P<0.001 versus control, n=10 mice in each group from two independent experiments. RGZ treatment resulted in a 46% increase in ABI as well as in a 30 and 25% decrease in MI and Ki-67 respectively compared to control group.
previously evaluated on xenograft tumor samples. H295R cells were treated with 20 μM RGZ for 24 h, and total mRNA was extracted and subjected to quantitative real-time RT-PCR analysis with the same primers used above. This RGZ concentration has already been used for in vitro experiments in H295 and H295R cells (Betz et al. 2005, Ferruzzi et al. 2005) and has been calculated to be the IC50 for RGZ inhibition of H295R cell proliferation (Cantini et al. 2008). Expression of the proliferation, angiogenic and anti-apoptotic markers, BMI-1, VEGF and Bcl-2 respectively, was significantly downregulated, while expression of seladin-1 was significantly increased by RGZ cell treatment (Fig. 4B), confirming the results obtained in the xenograft model.

Discussion

This paper reports, for the first time, the inhibitory effect exerted in vivo by RGZ on adrenocortical cancer growth in a xenograft model of human ACC obtained by s.c. injection of H295R in athymic CD1 mice. In particular, we demonstrate that 4 week oral administration of 5 mg/kg per day RGZ significantly inhibits tumor growth (1−T/C = 74.5%) and vascularization by reducing cell proliferation (significant reduction of MI and Ki-67 staining in RGZ versus control group) and stimulating apoptosis (significant increase in ABI in RGZ versus control group). These histological findings have been confirmed at molecular levels by the ability of RGZ to reduce the expression of angiogenic/vascular markers such as VEGF and CD31, of proliferation markers such as BMI-1 and of the anti-apoptotic marker, Bcl-2. The in vivo effects of RGZ in mouse xenografts have been confirmed by analogous findings obtained in vitro in RGZ-treated H295R.

Previous studies demonstrated that TZD inhibits in vitro proliferation of the human ACC cell model, H295R. In particular, RGZ reduces H295R cell growth in vitro with a calculated IC50 of 20 μM (Cantini et al. 2008), by interfering with the insulin-like growth factor receptor-1 axis and inducing cell differentiation and apoptosis (Betz et al. 2005, Ferruzzi et al. 2005, Cantini et al. 2008). RGZ anti-neoplastic effects have been observed in several types of cancer cells and in xenograft tumors (Keshamouni et al. 2004, Yoshizumi et al. 2004, Annicotte et al. 2006, Dai et al. 2008), although it is not yet clear whether RGZ action is mediated by PPARγ. Of interest, we found no alterations in PPARγ expression in xenograft tumors following RGZ treatment.

Our results indicate that RGZ anti-proliferative effects involve a downregulation of angiogenic and an upregulation of apoptotic genes both in vivo and in vitro, resulting in a significant reduction in vascularization and proliferation and an increase in apoptosis in RGZ-treated xenograft tumors. Gene expression inhibition by RGZ, as fold increase over controls, seems more evident in xenograft tumors than in in vitro treated cells, suggesting that RGZ effect is not only acting on H295R but also on the murine vascular component of the tumor. Indeed, all primers used, except for CD31 which is mouse-specific, can detect the expression of both human and mouse genes. Interestingly, RGZ inhibitory effects on gene expression are specific, since expression of seladin-1 is not inhibited but is even stimulated by RGZ treatment, in particular in in vitro experiments. Since seladin-1 is involved in the synthesis of cholesterol, the precursor of steroid hormones produced by the adrenal cortex, and since it is expressed at lower levels in malignant ACC than in normal adrenal gland (Luciani et al. 2004), it may be hypothesized that its increased expression in xenograft tumor and in H295R following RGZ, possibly associates with induced differentiation and reduced malignancy. The reduced expression of the polycomb gene Bmi-1 in RGZ-treated xenograft ACC not only confirms inhibition of proliferation, but also suggests that RGZ interferes with malignancy. In fact, in both lymphomas (van Kemenade et al. 2001) and breast cancer (Datta et al. 2007), BMI-1 emerged to be associated not only with proliferation markers such as Ki-67, but also with the degree of malignancy, thus representing a possible prognostic factor (Silva et al. 2007). This is the first time that BMI-1 expression has been studied in ACC. Further investigations are required to define the correlation between BMI-1 expression and ACC differentiation and malignancy.

Although in preclinical studies TZD showed anti-proliferative, pro-apoptotic, and differentiating effects, their use in clinical trials planned for the treatment of limited cohorts of patients affected by different types of solid tumors gave controversial and not encouraging results (Demetri et al. 1999, Debrock et al. 2003, Vogt et al. 2003, Reichle et al. 2004, Smith et al. 2004, Yee et al. 2007, Read et al. 2008). Indeed, only a few studies found TZD to exert some positive therapeutic effect, particularly if combined with chemotherapeutic and angiostatic drugs (Vogt et al. 2003, Reichle et al. 2004). This is of particular notice, since RGZ has been demonstrated to inhibit primary tumor growth and metastasis in different xenograft tumors through suppression of angiogenesis (Panigrahy et al. 2002).
Also in our ACC model, RGZ, at even lower doses, reduced the vessel number as well as the expression of endothelial and angiogenic markers. However, all the clinical studies so far reported have enrolled a limited number of patients and are too heterogeneous in terms of type and stage of tumors, patient’s age and sex and type of treatment (TZD doses, combined therapy, duration) to allow a solid analysis of TZD anticancer effects. Interestingly, Monami’s meta-analysis (Monami et al. 2008) found a significantly lower incidence of malignancies in RGZ-treated diabetic patients, suggesting a protective effect of RGZ versus specific cancers, such as gastro-intestinal and lung cancer.

The different RGZ effects observed in clinical and pre-clinical studies may be due to several factors, such as the duration of treatment and the doses of RGZ. The 8 mg/day oral administration of RGZ to diabetic patients, resulting in 1.3 μM RGZ circulating level, is the maximal dose reached in cancer studies and is far lower than the doses exerting anti-neoplastic effect in vitro (20–50 μM) and in xenograft tumors (50–150 mg/kg per day, Heaney et al. 2002). Thus, in an attempt to evaluate its true potential anticancer effects, it might be useful to increase RGZ doses to those which have been found efficacious in preclinical studies. Concerns about the potential cardiovascular risks (Nissen & Wolski 2007) of using RGZ at doses higher than 8 mg/day should be inconsistent since, at variance with diabetic subjects, ACC patients seldom suffer from cardiovascular or renal complications. Moreover, an overall well-tolerated RGZ administration was reported in patients with Cushing’s disease (Pecori Giraldi et al. 2006) or solid tumors (Read et al. 2008) at doses (12 and 16 mg/die for up to 8 months) higher than those used in diabetes treatment. Of note, we obtained a 70% inhibition of xenograft tumor growth with a RGZ dose 30 time lower than those currently used in xenograft studies (Heaney et al. 2002), without observing body weight loss or toxicity during the treatment. This result is notable, since a tumor inhibition percentage (1 − T/C) ≥ 60% has been defined for drug anticancer efficacy (Teicher 1998).

However, pharmacokinetic studies in the animals should be set up in order to evaluate which are the RGZ serum levels achieved in orally treated xenograft mice, which result in tumor growth inhibition in vivo.

Before hypothesizing a possible therapeutic use of RGZ and other TZD in ACC treatment, pharmacokinetic studies are mandatory to establish whether oral doses capable of resulting in around 20 μM circulating concentrations possess toxic effects in vivo in humans.

In conclusion, this is the first study demonstrating that RGZ effectively inhibits tumor growth in a human xenograft ACC model. These findings suggest a potential role for RGZ as a novel and promising adjuvant therapy after surgical removal of ACC, alone or in combination with mitotane to obtain more efficacious antitumor results. Further studies are needed to test the effect of a combined treatment of RGZ and mitotane in vitro and in vivo.

Declaration of interest
The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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