Inhibition of nuclear factor kappa B attenuates tumour progression in an animal model of renal cell carcinoma

Christudas Morais1,2, Helen Healy1, David W. Johnson3 and Glenda Gobe2,3

1Department of Renal Medicine, Royal Brisbane and Women’s Hospital, Herston, Queensland, Australia, 2Discipline of Medicine, School of Medicine, University of Queensland, Brisbane, Queensland 4006, Australia and 3Department of Renal Medicine, University of Queensland at Princess Alexandra Hospital, Brisbane, Queensland 4102, Australia

Correspondence and offprint requests to: Christudas Morais; E-mail: c.morais@uq.edu.au

Abstract

Background. Renal cell carcinoma (RCC) is a highly metastatic and lethal disease with few efficacious treatments. Many studies have shown that the ubiquitous transcription factor nuclear factor kappa B (NF-κB) plays a key role in the development and progression of many cancers including RCC. The aim of this investigation was to evaluate the anti-cancer effect of pyrrolidine dithiocarbamate...
PDTC decreases RCC growth

(PDTC), a NF-κB inhibitor, in a murine xenograft model of RCC.

Methods. The metastatic human RCC cell line, SN12K1, was inoculated into the left kidneys of severe combined immunodeficiency mice and the effect of semi-continuous PDTC treatment (50 mg/kg) on RCC growth analysed 5 weeks later. The analyses carried out in three groups (no treatment, RCC alone and RCC + PDTC) at 5 weeks were: renal weight, protein expression by immunohistochemistry and Western immunoblot, apoptosis (TdT-mediated nick end labelling and morphology) and mitosis (morphology).

Results. PDTC significantly decreased RCC growth and the expression of NF-κB subunits (p50, p52, c-Rel and RelB), upstream IKK-β and IKK-γ, but did not induce any changes in the expression of IκB-α and IκB-β. RCC growth was associated with a significant decrease in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, and increase in pro-apoptotic Bax, all of which were reversed by PDTC. Cell proliferation was significantly reduced by PDTC.

Conclusion. The results demonstrate the potential anti-cancer benefits of treating NF-κB positive RCCs with NF-κB inhibitors like PDTC.

Keywords: apoptosis; NF-kappa B; pyrrolidine dithiocarbamate; renal cell carcinoma

Introduction

Renal cell carcinoma (RCC), the most common type of kidney cancer, is among the top 10 leading causes of cancer-related deaths worldwide [1,2]. RCC is a highly metastatic disease, and metastatic RCC is essentially incurable and lethal. The average survival of patients, following metastatic RCC, is about 4 months and only 10% of patients survive for 1 year [3,4]. None of the currently available chemotherapy, radiotherapy, hormonal or biological therapies is significantly more efficacious than placebo in the treatment of metastatic RCC. Some of the major impeding factors in the effective treatment of metastatic RCC include systemic toxicity of chemotherapeutics to normal cells, resistance of RCC cells to chemotherapy-induced apoptosis, high rate of angiogenesis and hyper-proliferation. Novel chemotherapeutics are urgently required for the treatment of this deadly disease.

Of the many molecules that have been implicated in the development and progression of RCC, the ubiquitous transcription factor nuclear factor kappa B (NF-κB) has attracted the attention of investigators in recent years. NF-κB is a collective term for dimeric transcription factors of the Rel family of DNA-binding proteins [5] that recognize a specific sequence called the κB site. There are five known members of the mammalian Rel family: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). In unstimulated states, NF-κB is sequestered in cytoplasm in an inactive form bound to one of many inhibitors, including IκB-α and IκB-β. The IκBs contain two conserved serine residues in their N-terminal domain. Upon stimulation, phosphorylation of the serine residues by the IκB kinase (IKK) complex, which consists of three subunits, IKK-α, IKK-β and IKK-γ, leads to the release, activation and translocation of active NF-κB to the nucleus. Here, it binds to κB elements and regulates transcription of genes involved in a variety of biological processes, including carcinogenesis [5-8]. NF-κB upregulates anti-apoptotic [9], pro-angiogenic [10] and multi-drug resistance pathways [11] which collectively impart a hyper-proliferative, anti-apoptotic and metastatic phenotype to RCC cells and to cancer cells in general. Overexpression of NF-κB has been reported in almost all cancers [12].

Pertinent to RCC, Oya and colleagues reported a direct correlation between tumour grade, invasion and metastasis of RCC and the expression and activation of NF-κB [13]. Subsequently, Sourbier and colleagues showed that clear cell RCCs, which contribute to nearly 75% of all RCC subtypes, have a higher expression of NF-κB than other subtypes of RCC [2]. These authors also reported a correlation between tumour dimension and NF-κB expression. Recently, two independent reports further substantiated the overexpression of NF-κB in human RCC samples [14,15], and many RCC cell lines have been shown to overexpress NF-κB [2,16-18]. In addition, nearly 50% of long-term dialysis patients will develop cystic changes of the kidneys and ~6% of these patients will develop RCC [19,20]. A recent report showed that overexpression of NF-κB may be involved in the development of renal cysts and their subsequent transformation into RCC [21]. Therefore, inhibition of NF-κB as a potential treatment for RCC has been actively explored over recent years. For example, inhibition of NF-κB using parthenolide [22], bortezomib [18], sulfazalazine and Bay-11-7085 [2] induced apoptosis in many RCC cell lines. In vivo, parthenolide [22] and Bay-11-7085 [2] have been shown to inhibit RCC growth in murine xenograft models. However, the in vivo studies have injected RCC cell subcutaneously into mice rather than into the kidney. To the best of our knowledge, in vivo studies that assessed the effect of NF-κB inhibition in kidneys with actively growing RCC have not been reported. In this study, we tested the effect of pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of NF-κB, in an in vivo model of RCC.

Materials and methods

Ethics approval

This research was approved by the Animal Experimentation Ethics Committee of the University of Queensland (AEEC #RBH/124/06/ARU).

Chemicals

Ammonium PDTC (Sigma-Aldrich, catalogue number P8765), MTI (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; catalogue number M2128), Ponceau S solution (catalogue number P7170), isopropanol (catalogue number L-9516) and 0.1 N hydrochloric acid (catalogue number H9892) were purchased from Sigma-Aldrich Pty Ltd (St Louis, MO, USA). The following tissue culture medium and supplements were purchased from Gibco (Invitrogen, CA, USA): 1:1 mixture of Dulbecco’s Modified Eagle’s Medium/Ham’s F12 (DMEM/F12; catalogue number 11330-057); foetal bovine serum (FBS, catalogue number 10099-141); antibiotic/antimycotic solution containing penicillin, amphotericin B and streptomycin (catalogue number 15240-062); and trypsin-EDTA (catalogue number 25300-026).
Protease inhibitors (complete mini; catalogue number 11 836 153 001) were purchased from Roche Diagnostics Corporation (IN, USA). Tissue culture flasks and culture dishes were purchased from Becton Dickinson Labware (NJ, USA). The sources of other materials are mentioned within the following text.

**Antibodies**

The following primary and secondary antibodies were obtained from Santa Cruz Biotechnology (CA, USA): Actin (sc-1615), Bax (sc-6263), Bel-2 (sc-492), Bcl-XL (sc-7195), Bcl-2α (sc-847), Bcl-2β (sc-945), IKK-α (sc-7182), IKK-β (sc-8014), IKK-γ (sc-8032), NF-κB p50 (sc-7178); NF-κB p52 (sc-298); NF-κB p65 (sc-372), c-Rel (sc-70); Rel B (sc-226), goat anti-rabbit IgG-HRP (sc-2004) and bovine anti-mouse IgG-HRP (sc-2371).

**Cell lines and culture**

The metastatic human RCC cell line SN12K1 was obtained from Professor D Nicol, Princess Alexander Hospital, Brisbane, Australia, through his collaborations with Dr I Fidler, Cancer Research Institute, MD Anderson Cancer Center, Orlando, FL. The SN12K1 cells were cultured in DMEM/F12 containing 10% FBS supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml) and amphotericin B (0.125 μg/ml) in a humidified atmosphere of 95% air and 5% CO₂.

**Cell viability assay using MTT**

Cell viability was assessed by an MTT assay. Cells (2 × 10⁴ cells/well/100 μl) were seeded in 96-well plates. The culture volume was 100 μl. Twenty-four hours later, 10 μl of MTT from a 5 mg/ml stock in phosphate-buffered saline (PBS) was added to each well of the culture plate and incubated for 90 min at 37°C. The culture medium was removed and the purple crystals formed were dissolved in 150 μl isopropanol containing 0.1 N hydrochloric acid. The absorbance was read at 570 nm with a background correction of 690 nm in a VERSAmax microplate reader (Selby Biolab, Australia).

**Trypan blue dye exclusion assay for cell viability**

Cell viability was assessed by a trypan blue exclusion assay. The cells were counted and suspended in normal culture medium. A 1:1 dilution of cell suspension was prepared using a 0.4% trypan blue solution (Sigma-Aldrich Pty Ltd, catalogue number T 8154). The cell suspension was loaded in a haemocytometer and the live cells were counted and calculated as per normal procedures.

**Animal model of renal cell carcinoma**

Adult male severe combined immunodeficiency (SCID) mice were obtained from the Animal Research Facility, Perth, Australia. The animals were acclimatized at the Herston Medical Research Facility, University of Queensland, Brisbane, Australia, through the experimenters were carried out. The animals were kept under 12 h dark/light conditions and food and water were given *ad libitum*. SN12K1 cells were grown as described above. On the day of the experiments, they were trypsinized and resuspended in PBS at a density of 1 × 10⁶ cells per 0.1 ml of PBS. The viability of the cells was verified using the trypan blue exclusion assay. The cell suspension was kept on ice until the time of injection. PDTIC was prepared in physiological saline at a concentration of 10 mg/ml. The animals were anesthetized with an i.p. injection of ketamine–xylazine solution (80 and 16 mg/kg, respectively). A flank incision was made on the left side of the animals of the RCC-alone and RCC+PDTIC groups, and 1 × 10⁶ cells in a dose volume of 100 μl were injected into the cortical parenchyma of the left kidney as previously described [24]. The wound was closed with 4.0 silk sutures and the animals were allowed to recover. Twenty-four hours after the surgery, the animals in the RCC+PDTIC group were given an i.p. injection of PDTIC at a dose of 50 mg/kg. This dose was maintained throughout the study. The PDTIC treatment regime was once daily for 5 days a week (continuous) for the first 2 weeks and then 4 days a week (continuous) until the end of the study. The animals in the RCC-alone group were given placebo of PBS.

The time interval from processing of cells from cell culture to their injection into the left kidney of the last animal was ~60 min. Because the method of processing, low temperature storage and the time delay to final injection had the potential to affect the viability of the cells, the number of viable cells was determined at the end of the procedure. Trypan blue dye exclusion assay showed that 95% (0.95 × 10⁶ per 0.1 ml) of the cell inocula were viable after this time period (data not shown). In order to verify the effect of processing on the growth rate of RCC cells, a comparative study was carried out between unprocessed cells and the processed cells. The cells from each procedure were seeded in 96-well plates (2 × 10⁵ cells/well/100 μl) and, 24 h later, cell viability was analysed by MTT assay. No significant differences between the viability of the processed cells and unprocessed cells were observed (data not shown).

A separate PDTIC alone group was not included. This decision was based on maintaining low animal numbers and previous findings that showed PDTIC, up to an i.p. dose of 250 mg/kg, was well tolerated in mice without any significant toxicity to normal tissues [25]. A dose of 100 mg/kg is routinely used in murine research as non-toxic to normal tissues [26–29]. The right kidneys from the RCC+PDTIC group served to study the effect of PDTIC on normal kidneys.

The body weight of the animals was monitored. Five weeks later, the animals were euthanized by cervical dislocation. All organs were checked macroscopically for formation of metastatic clones of the RCC. The kidneys were harvested and the peri-renal fat removed, and then the kidneys were weighed and photographed. The kidneys were then sliced lengthwise, ensuring even distribution of any tumour material. Half was collected for immunohistochemistry and half for protein extraction and analysis. For immunohistochemistry, the tissues were fixed in 4% buffered formaldehyde, transferred to 75% ethanol after 24-h fixation at 4°C and kept in this solution until further routine histological preparation. For protein extraction, the tissues were minced at 4°C using a pair of precooled forceps. A portion of the minced tissues was frozen immediately in dry ice and stored at −80°C until further use. Another portion was collected in ice-cold 1× hypotonic buffer for the isolation of nuclear proteins.

**Western blotting**

Nuclear proteins from renal tissue extracts were prepared using a commercially available nuclear protein extraction kit (Active Motif, Carlsbad, CA, USA; catalogue number 40010). For whole tissue lysates, tissues were thawed in ice-cold radio immunoprecipitation assay (RIPA) buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing complete protease inhibitor cocktail. The tissues were thoroughly mashed using a pair of forceps, and the tissue–RIPA mixture was kept on ice for 30 min then centrifuged at 13,000 r.p.m. for 15 min at 4°C. The supernatant which represented the total tissue lysate was collected. The collected supernatants were further centrifuged twice as described above. The protein content of the nuclear lysates and whole tissue lysates was measured using biocinchonic protein assay reagent (Pierce, Rockford, IL, USA). The lysates were aliquoted and stored at −80°C until further use. The proteins were resolved in 12% Tris-HCl gel (Biorad, Hercules, CA, USA) and electro-transferred into Hybond-C nitrocellulose membrane (Amersham Biosciences, UK). Equal transfer and loading of proteins was further confirmed by staining the membranes with Ponceau S solution. Standard Western blotting procedures were followed, and the proteins were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce). The differences in intensities of the signals were analysed by Scion Image software (Scion Corporation, MD, USA).

**Transcription activity of NF-κB subunits**

DNA binding activity as a measure of transcription activity of NF-κB was analysed using a commercially available enzyme-linked immunosorbent assay-based TransAM NF-κB family assay kit (Active Motif, Carlsbad,
A developing solution was added and observed for the development of blue

Fixed tissue was embedded in paraffin and sectioned at 3

Immunohistochemistry of kidney samples

The preparation of reagents and the assay were carried out as per the in-
structions of the supplier. Nuclear proteins (5 µg) were added to each well
and incubated at room temperature for 1 h. During this process, the active
NF-κB in the nuclear extract specifically binds to the NF-κB consensus
sites immobilized in 96-well plates. The wells were washed using the
washing buffer and incubated with respective primary antibodies which
recognize an epitope on p50, p52, p65, c-Rel or RelB that is accessible
only when NF-κB is activated and bound to its target DNA site, immob-
ilized in 96-well plates. The wells were washed and incubated with
horseradish peroxidase (HRP)-conjugated secondary antibodies and incu-
bated at room temperature for 1 h. After washing the wells, 100 µl of
developing solution was added and observed for the development of blue
colour. The reaction was stopped by adding 100 µl of stop solution, and
the absorbance was read using a microplate reader at 450 nm with a ref-
ence wavelength of 655 nm.

Immunohistochemistry of kidney samples

Fixed tissue was embedded in paraffin and sectioned at 3–4 µm thickness
onto Superfrost® Plus slides (Menzel-Glasser, Braunschweig, Germany)
by staff at the Histology Services Unit, Queensland Institute of Medical
Research, Brisbane, Australia. Sections were de-paraffinized in xylene
(3 × 5 min) in TBS and then incubated with the appropriate HRP-conjugated
secondary antibody for 30 min at room temperature. Sections were
then washed (3 × 5 min) in TBS, developed with 3,3′-diaminobenzidine for 2–3
min, lightly counterstained with haematoxylin, and mounted with cover-
slips using DePex mounting medium (Searle Diagnostics, High Wy-
combe, Bucks, UK) for light microscopy and photography. Sections
were batch-stained for each antibody. Negative controls (omitting pri-
mary antibody or using horse serum) were used with each batch. Po-

ditive controls were used in instances where there appeared to be no
reactivity of antibody in the test sections to test if the antibodies were
active.

Haematoxylin and eosin staining for morphology, apoptosis and prolifera-
tion

The de-paraffinized and rehydrated tissue sections were washed two to
three times with PBS and then incubated in 0.2 µm filtered Mayer’s
haematoxylin stain (Sigma-Aldrich; catalogue number MHS32) for 5–
10 min. The haematoxylin was ‘blued’ in normal tap water for 2 min or
with ‘bluing’ agent (0.2% [w/v] aqueous sodium carbonate) for a further
5 min to intensify the stain. Tissue sections were immersed in 70% eth-
anol and then stained with alcoholic eosin solution (Sigma-Aldrich; cat-
ologue number 11983-0) for 5 min, followed by 2 × 2–3 min washes in
100% ethanol. Tissue sections were cleared in 2 × 2 min washes in xyl-
en. Coverslips were mounted onto slides using DePex mounting medi-
um (Searle Diagnostics) for light microscopy and photography. The cells
were viewed under ×40 objective (∼400 magnification), and the apoptotic
and proliferating cells that fell within the 100 squares of an eyepiece grat-
icule were counted. Apoptotic nuclei display certain distinct morphologic
features [31–34]: hyperchromasia; shrunken/condensed nuclei, blebbing
of the membrane whilst maintaining membrane integrity, crescent nuclei
and apoptotic bodies. Proliferating cells were identified by the mitotic
plate and cytokinesis. The number of apoptotic and proliferating cells
was expressed as a percentage of the total cells counted from five random
fields for each slide.

Results

PDTC prevented body weight loss and reduced RCC growth

There was no significant difference in the initial body weights of animals between the groups (Figure 1A). Throughout the experiments, the animals in the RCC-alone group consistently lost body weight. From Week 4, the body weights of animals in the RCC-alone group were significa-

![Fig. 1. Assessment of body weight and kidney weight. PDTC prevented body weight loss (A) and decreased the growth of RCC (B). *P<0.05, **P<0.01 and ***P<0.001 vs control group; #P<0.05 and ##P<0.01 vs RCC-alone group.](image-url)
Although the body weight of the animals in the RCC+PDTC group remained lower than the control animals, the difference was not statistically significant (Figure 1A). The effect of PDTC on the growth of the RCC was analysed by weighing the left kidneys. The weights of the left kidneys of the RCC-alone group were significantly higher than the weight of the left kidneys of the control or RCC+PDTC groups (Figure 1B). Treatment with PDTC significantly limited the tumour-induced increase in the left kidney weight (Figure 1B). The effect of PDTC was further evaluated on the right kidneys (i.e. the kidneys that were not injected with RCC cells). The kidneys in the RCC+PDTC group showed a slight, non-significant increase in weight when compared with the right kidneys of the control or RCC-alone group (Figure 1B).

**Macroscopic and microscopic characteristics**

Macroscopically, the right kidneys of the RCC+PDTC group appeared slightly larger, but without any changes in colour or consistency, compared to the right kidneys of the control or RCC-alone groups (Figure 2; first and second panels from left; kidneys labelled ‘right’). In the left kidneys of the RCC-alone group, no macroscopically visible ‘normal’ areas could be identified in any of the four kidneys (Figure 2; first panel from left; kidneys labelled ‘left’). PDTC treatment significantly reduced the growth of RCC. ‘Normal’ areas, which could be identified by the characteristic dark brown colour of the kidneys, were macroscopically visible (Figure 2; second panel from left; arrows; kidneys marked ‘left’). When longitudinally cut open, the kidneys in the RCC-alone group were replaced by yellow masses of tumour growth (Figure 2; third panel from left; kidneys marked ‘left’). In the RCC+PDTC group, although there was tumour growth, the structural integrity of the kidneys was maintained with the presence of more normal regions that had no visible signs of RCC growth when compared with the RCC-alone group (Figure 2; fourth panel from left; arrows; kidneys marked ‘left’).

Haematoxylin and eosin staining of sections of the cortical and medullary regions of the right kidneys showed no significant structural changes among groups (data not shown). In the left kidneys of the RCC-alone group, no normal structure, either in the cortex or medulla, could be observed (Figure 3; second panel from top). There were varying degrees of changes, mostly infiltration of the kidney by the RCC cells replacing the normal structures with patchy fibrotic and necrotic masses (Figure 3; second panel from top). In the RCC+PDTC group, although there were changes similar to that of the RCC-alone group, there were also normal areas devoid of any cancer growth (Figure 3; third panel from top). In addition, in the kidneys of RCC+PDTC group, there were tumour–normal interface areas (Figure 3; fourth panel from top), in which normal structures were surrounded by or interspersed with areas of RCC growth.

**Effect of PDTC on expression of NF-κB subunits**

The expression of the NF-κB subunits in the tissue lysates of left and right kidneys was analysed by Western blotting. Western blots of the whole cell lysates of the left kidneys

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**Fig. 2.** Morphology of kidneys. PDTC decreased the growth of RCC in the left kidneys whereas no changes were observed in the right kidneys among the groups.
showed that, of the five subunits, p65 had the strongest expression in control kidneys. There was a weak expression of the p50 subunit, and the expression of p52, c-Rel and RelB was almost non-detectable in the control kidneys (Figure 4A, lane 1). In the RCC-alone group, the expression of all subunits except for p65 was significantly higher than the control group (Figure 4A, lane 2). In the RCC+PDTC group, all subunits, except for p65, were significantly reduced when compared with the RCC-alone group (Figure 4A, lane 3). NF-κB expression in the whole cell lysates of the right kidneys was comparable to that of the control left kidneys (data not shown).

The expression of NF-κB subunits in the left and right kidney sections were further analysed by immunohistochemistry. Similar to the Western blots, no marked expression of the NF-κB subunits, except for p65, could be observed in the control sections (Figure 4B; first panel from left). In the RCC-alone group, the NF-κB subunits were cytoplasmic and the intensity of the staining was stronger than the control group (Figure 4B; second panel from left). In the RCC+PDTC group, the expression of the NF-κB subunits was decreased when compared with the RCC-alone group (Figure 4B; third panel from left). However, this comparison was made between the normal areas in renal sections of the RCC+PDTC group and the tumour area of the RCC-alone group. Therefore, in order to assess the direct effect of PDTC on NF-κB expression, a comparison was made between the tumour areas of the RCC+PDTC group and the tumour areas of the RCC-alone group. The results showed that, in the tumour areas of the RCC+PDTC group, the intensity of NF-κB staining was less than that of the tumour areas of the RCC-alone group (Figure 4B; fourth panel from left). Immunohistochemistry of right kidneys showed that the expression patterns of the NF-κB subunits were comparable to that of the control left kidneys (data not shown). No marked differences in the expression of NF-κB were observed among the three groups.

**Effect of PDTC on the NF-κB transcription and expression of IKK complex**

NF-κB transcription activity was measured in the nuclear lysates of the left kidneys. Of the five subunits, p65 showed the strongest activity in the control kidneys (Figure 5A). In the RCC-alone group, the transcription activity of all subunits, except for p65, was significantly higher than the control group (Figure 5A). In the RCC+PDTC group, the transcription activity of the subunits, except for p65, was significantly reduced when compared with the RCC-alone group (Figure 5A).

The expression of the IKK complex (IKK-α, IKK-β and IKK-γ) and the inhibitory units (IκB-α and IκB-β) was analysed by Western blotting. Of the three subunits of the IKK complex, IKK-α showed the strongest expression, whereas the expression of IKK-β and IKK-γ was very weak in the control kidneys (Figure 5B, lane 1). In the RCC-alone group, significant increases in the IKK-β and IKK-γ but no significant changes in IKK-α were observed when compared with the controls (Figure 5B, lane 2). In the RCC+PDTC group, the expression of IKK-β and IKK-γ was significantly reduced when compared with the RCC-alone group (Figure 5B, lane 3). Although the expression of IKK-α was reduced in the RCC+PDTC group when compared with the RCC-alone group, this was not found to be statistically significant. The expression of the IKK complex in the whole cell lysates of the right kidneys was comparable to that of the control left kidneys (data not shown).

Western blots of left kidneys showed that both IκB-α and IκB-β were expressed in control kidneys (Figure 5B, lane 1). No significant changes in the expression of the IκBs were observed either in RCC-alone or RCC+PDTC group when compared with the controls (Figure 5B, lanes 2 and 3). The expression of the IκBs in the whole cell lysates of the right kidneys was comparable to that of the control left kidneys (data not shown).

**Effect of PDTC on apoptosis-regulatory proteins, apoptosis and proliferation**

We previously reported that induction of apoptosis and inhibition of proliferation of SN12K1 were the major mechani-
isms of action of PDTC in vitro [23]. Whether or not similar mechanisms were involved in the in vivo activity of PDTC was explored. Since the left kidneys of the RCC+PDTC group had normal, non-RCC-affected regions in the kidney, and these regions were devoid of significant apoptotic or proliferating cells, the analyses of apoptosis and proliferation were cautiously assessed. Analysis of apoptotic and proliferating cells from randomly selected areas of whole sections would bias results in favour of the PDTC group. Therefore, two independent analyses were made. In the first
analysis, apoptotic and proliferating cells from five random
areas (one in the middle and four from the periphery assum-
ing four corners of a square) of each kidney section were
made. In the second analysis, the number of apoptotic and
proliferating cells was recorded only from the tumour areas
(five different areas) of the RCC-alone and the RCC+
PDTC groups. The first analysis found a significant in-
crease in the number of apoptotic and proliferating cells in
the RCC-alone group when compared with the control
group (Figure 6A–B; whole section). In the RCC+PDTC
group, the number of apoptotic and proliferating cells was
significantly less than the RCC-alone group (Figure 6A–B;
whole section). However, when the results were analysed
using the second approach, no significant differences in
the number of apoptotic or proliferating cells were observed
between the tumour areas of the RCC-alone or the RCC+
PDTC groups (Figure 6A–B; tumour area). The right
kidneys did not show significant alteration in apoptosis or
proliferation over the background levels reported for the
control left kidneys. No significant differences in apoptosis
or proliferation were observed among the three groups (data
not shown).

Next, the expression of the apoptotic regulatory proteins
in the left and right kidneys was analysed by Western blot-
ing. Of the three groups, the control left kidneys showed the
highest expression of the anti-apoptotic proteins Bcl-2 and
Bcl-xL. The expression of these proteins was significantly
decreased in the left kidneys of the RCC-alone groups when
compared with the control group (Figure 6A–B; whole section). However, when the results were analysed
using the second approach, no significant differences in
the number of apoptotic or proliferating cells were observed
between the tumour areas of the RCC-alone or the RCC+
PDTC groups (Figure 6A–B; tumour area). The right

Fig. 5. Assessment of NF-κB transcription activity, IKK complex and IκBs. PDTC decreased the transcription activity of NF-κB subunits (A) and IKK-
β and IKK-γ (B), but not IκB-α and IκB-β (lane 1, control; lane 2, RCC; lane 3, RCC+PDTC). Histograms below the Western blots (B) show
quantitative densitometry of each protein. *P<0.05 and ***P<0.001 vs control group; #P<0.05; ##P<0.01 and ###P<0.001 vs RCC group.
pression of Bax was significantly increased when compared with the control group (Figure 6C). By comparison, Bax was significantly decreased in the RCC+PDTC group (Figure 6C). The expression of Bcl-2, Bcl-XL and Bax in the right kidneys of the three groups was similar to that of the control left kidneys. No significant differences in the expression of these proteins were observed among the right kidneys of the three groups (data not shown).
In order to substantiate these results, immunohistochemistry of the left and right kidneys was performed. Bcl-2 and Bcl-\text{xL} expressions were confined to normal tubules. The left kidneys of the control groups showed a higher basal level expression of Bcl-2 and Bcl-\text{xL} (Figure 6D; first panel from left). The expression of Bcl-2 and Bcl-\text{xL} was markedly decreased in the RCC-alone group when compared with the basal expression of the control group (Figure 6D; second panel from left). In the RCC+PDTC group, the expression of these proteins was increased when compared with the RCC-alone group (Figure 6D; third panel from left).

These proteins are expressed in the normal tubules with structural integrity. Such tubules were lacking in the RCC-alone group. It is questionable whether the expression of these proteins in structurally sound tubules (normal area) could be compared with tubules that have been infiltrated with RCC and in many cases have become degraded and necrotic. Therefore, as in the case of apoptosis, a comparison of intensity of staining was performed between the tumour areas of the RCC-alone group and the tumour areas of the RCC+PDTC group. No significant differences in the intensity of staining were observed between the tumour areas of the RCC-alone group and the tumour areas of the RCC+PDTC group (Figure 6D; fourth panel from left).

Immunohistochemistry of right kidneys showed that the expression of Bcl-2 and Bcl-\text{xL} was similar to the left kidneys of the control group. No significant differences in the expression of these proteins were observed among the right kidneys of the three groups (data not shown).

Metastasis

Of the animals that were inoculated with RCC, two animals in the RCC-alone group developed metastasis in the small intestine, which were evident by the presence of tumour nodules (data not shown). No lesions were observed in any of the RCC+PDTC group (data not shown).

Discussion

The results presented in this study demonstrate the anti-cancer properties of PDTC in an RCC murine xenograft model. PDTC prevented body weight loss and RCC progression in mice. Importantly, PDTC was selectively toxic to RCC and did not induce any pathological changes in the normal kidney cells. The internal organs of the PDTC-treated animals appeared normal upon visual inspection. The implanted RCC cells maintained their NF-\text{\kappa}B expression and their transcription activity. This was evident from the weak expression and transcription activity of NF-\text{\kappa}B subunits, except for p65, in control kidneys when compared with the higher expression of NF-\text{\kappa}B subunits and transcription activity in RCC-implanted kidneys.

PDTC treatment significantly decreased the expression and transcription activity of NF-\text{\kappa}B subunits. It also decreased the expression of IKK-\text{\beta} and IKK-\text{\gamma}, in line with previous reports [23,35]. The release of active NF-\text{\kappa}B from the inhibitory molecules such as I\text{\kappa}B-\alpha and I\text{\kappa}B-\beta is mediated by IKKs through the classical, alternate or atypical pathways. In the classical pathway, the IKK-\text{\beta} subunit is activated, which in turn phosphorylates the I\text{\kappa}B-\alpha subunit at specific serine residues and releases the active NF-\text{\kappa}B [5,7]. In the alternative pathway, IKK-\alpha is activated, thereby cleaving the inactive p100 molecule and generating the active p52 subunit, which subsequently is translocated to the nucleus [36]. IKK-\gamma, although not known to be directly involved in the degradation of I\text{\kappa}B\text{s}, is essential for the assembly of the IKK complex and the activation of IKK-\alpha and IKK-\text{\beta} [5,7,36]. PDTC acted at multiple points of the NF-\text{\kappa}B signalling pathway and inhibited the IKKs, NF-\text{\kappa}B subunits and their transcription. It is possible that PDTC-mediated inhibition of the NF-\text{\kappa}B pathway resulted in lower proliferation of the RCC populations. This conclusion is supported by the extent of tumour growth in the RCC-alone and RCC+PDTC kidneys. In the RCC-alone group, the whole kidneys were occupied by tumour without sparing any normal cells. In the RCC+PDTC group, there was still cancer growth, indicating that PDTC did not prevent the growth of RCC. Rather, PDTC appears to have reduced the proliferation rate of the implanted RCC cells, thereby permitting survival of normal tissues in this group.

While the results are in favour of the anti-cancer properties of PDTC and the beneficial effect of NF-\text{\kappa}B inhibition in RCC, the interpretation of the molecular mechanism is very challenging. Our in vitro studies demonstrated that PDTC had a direct inhibitory effect on the NF-\text{\kappa}B subunits and their transcription activity and that it directly induced apoptosis and inhibited proliferation of SN12K1 cell lines [23]. It is doubtful such interpretation of these parameters can be made about the observed in vivo changes. It should be noted that there was a weak expression of the NF-\text{\kappa}B subunits in the control kidneys. In the RCC-alone group, the expression of these proteins was significantly increased when compared to the control kidneys. RCC cells had to be the source of these proteins because no normal cells could be identified in these.

Fig. 6. Assay of apoptosis, cell proliferation and apoptotic regulatory proteins. Analysis of apoptosis (A) and proliferation (B) from five randomly selected areas of whole kidney sections showed a significant increase in the number of apoptotic and proliferating cells in the RCC-alone group, which was less in the RCC+PDTC group (left panel). When the comparison was made between the tumour areas of the RCC-alone and the RCC+PDTC groups, no significant difference was observed (right panel). Western blots showed that the RCC-alone group had significantly lower expression of the anti-apoptotic proteins Bcl-2 and Bcl-\text{xL} and higher expression of the pro-apoptotic Bax when compared with control (C; lane 1, control; lane 2, RCC; lane 3, RCC+PDTC). In the RCC+PDTC group, the expression of Bcl-2 and Bcl-\text{xL} was increased and Bax was decreased when compared with the RCC-alone group (C). Histograms below the blots show quantitative densitometry of each protein. The expression pattern of Bcl-2 and Bcl-\text{xL} was further confirmed by immunohistochemistry (D). The third panel from left shows Bcl-2 and Bcl-\text{xL} expression in the normal areas of the RCC+PDTC group, whereas the fourth panel from left shows Bcl-2 and Bcl-\text{xL} expression in the tumour area of the RCC+PDTC group (D). No marked differences in the expression of Bcl-2 and Bcl-\text{xL} were observed between the tumour areas of RCC-alone group and RCC+PDTC group. *P<0.05 and ***P<0.001 vs control group; #P<0.05 and ####P<0.05 vs RCC-alone group.
kidneys either macroscopically or microscopically. The kidneys in the RCC+PDTC group comprised a mix of RCC and normal cells. Therefore, it could be argued that the decrease of NF-κB in the RCC+PDTC group was a reflection of more normal tissues in this group rather than a direct effect of PDTC on NF-κB inhibition. While this argument cannot be ruled out, comparative observation of the immunohistochemistry in the sections showed that the intensity of the NF-κB staining in the tumours of RCC+PDTC-treated kidneys was less than in the tumours of RCC-alone kidneys. It is reasonable to conclude that the observed changes in the expression pattern of molecules develop from a combination of the direct effect of PDTC as well as the presence of normal kidney components in the RCC+PDTC group.

In order to elucidate the mechanism of NF-κB-mediated decrease in cancer progression, we analysed the expression of Bel-2 and Bel-XL, which are considered to be under the transcriptional control of NF-κB. NF-κB acts as a pro-survival factor for cancer cells by triggering the expression of these anti-apoptotic molecules [37–40]. Pertinent to RCC, a correlation between increased expression of NF-κB and Bel-2 has been reported [15]. Furthermore, Bel-2 is overexpressed in RCC [41–43] and confers resistance to chemotherapy [44,45]. The inhibition of NF-κB has been shown to exert anti-cancer effects in many cancer types through the down-regulation of Bel-2 and Bel-XL [46,47]. Contrary to these findings, in our study, the tumour areas of the RCC-alone group showed a decrease in Bel-2 and Bel-XL expression despite an increase in NF-κB. Furthermore, in the RCC+PDTC group, despite a decrease in NF-κB, no marked difference in the expression of the above anti-apoptotic proteins in the tumour area was observed when compared with the tumour area of the RCC-alone group. The reason for this paradox is not clear and warrants further research. However, the observed changes in the expression pattern of the anti-apoptotic proteins Bel-2 and Bel-XL and the pro-apoptotic protein Bax seem to offer a possible explanation for the mechanism of RCC progression. For example, it has been reported that T cells isolated from RCC patients are highly susceptible to apoptosis [48,49]. Also, co-culture of T cells with RCC cells has been shown to induce apoptosis in T cells [50,51]. Further studies have shown that the mechanism behind the induction of T-cell apoptosis by RCC is through the down-regulation of Bel-2, and transfection of T cells with Bel-2 protected them from RCC-induced apoptosis [49,51]. As SCID mouse does not have functional T cells, our study suggests that down-regulation of Bel-2 by RCC is not only limited to T cells but also to normal renal cells. Therefore, it can be speculated that when RCC progresses and invades adjacent regions of the kidney, there is a destabilization and down-regulation of the inherent anti-apoptotic proteins Bel-2 and Bel-XL and up-regulation of the pro-apoptotic protein Bax. PDTC, on the other hand, through the inhibition of NF-κB, attenuates the progression of RCC and indirectly helps maintain the balance between the pro-apoptotic and anti-apoptotic proteins of normal cells.

In summary, PDTC is clinically well tolerated [52,53] and has been shown to offer beneficial effects in a variety of experimental models including cerebral ischaemia [53], chronic inflammation [54], septic shock syndrome [55] and retinal neovascularization [56]. PDTC also has antiviral activity against human influenza virus [57]. Earlier studies reported anti-cancer activity in many cancer cell lines. These include PDTC-induced cell death in breast [58], gastric [59], melanoma [60], ovarian [61], pancreatic [62] and prostate [63] cancer cell lines. PDTC prevented cancer cachexia in tumour-bearing mice [29]. In addition, PDTC has been shown to enhance the cytotoxicity of the anti-cancer drugs paclitaxel in ovarian cancer cell lines [61]. In this study, we further substantiate the anti-cancer effect of PDTC and the beneficial effect of NF-κB inhibition in an animal model of RCC. RCCs are a heterogeneous group of diseases with at least 16 known subtypes [64]. Each subtype may respond differently to a particular treatment. In this study, we report the anti-cancer effect of PDTC on SN12K1 cells. Further studies using different RCC cell types are warranted to fully explore the potential of PDTC as an anti-cancer agent against RCC.

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References


63. Pyrrolidine dithiocarbamate (PDTC) in suppressing inflammation in mice increases the therapeutic index of 5-fluorouracil in a mouse model.


100: 438


48. Yu, Y, Y, Y, Y et al. Growth inhibitory effects of obovatol on prostate and colon cancer cell lines. 

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13. Kerr JF, Gobe GC, Winterford CM


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Does endothelin B receptor deficiency ameliorate the induction of peritoneal fibrosis in experimental peritoneal dialysis?

Philipp Kalk¹,², Matthias Rückert², Michael Godes¹, Karoline von Websky¹, Katharina Relle¹,³, Hans-Hellmut Neumayer², Berthold Hocher¹,²* and Stanislao Morgera²,*

¹Center for Cardiovascular Research/Department of Pharmacology and Toxicology, Charite, Berlin, Germany, ²Department of Nephrology, Charite, Berlin, Germany and ³Institute of Vegetative Physiology, Charite, Berlin, Germany

Correspondence and offprint requests to: Stanislao Morgera; E-mail: Stanislao.morgera@charite.de

*Both authors contributed equally to the publication.

Abstract

Background. Peritoneal fibrosis is a serious complication of peritoneal dialysis (PD); however, the mechanisms are poorly understood. The endothelin system exhibits potent pro-fibrotic properties and is known to be stimulated in peritoneal fibrosis. Thus, our study aimed at elucidating the impact of the endothelin B (ETB) receptor on peritoneal membrane thickening by means of an ETB-deficient rat model (ETB⁻/⁻) in experimental PD.

Methods. Wild-type (WT) and ETB⁻/⁻ rats were randomly allocated to four groups (each group n = 10): (i) WT Sham, (ii) WT PD, (iii) ETB⁻/⁻ Sham and (iv) ETB⁻/⁻ PD. All animals underwent surgical implantation of a port for intraperitoneal administration and 1 week of habituation to the procedure by administration of 2 ml of saline once daily. Afterwards, all animals were switched to 12 weeks of 15 ml of saline (Sham groups) or commercially available PD fluid containing 3.86% glucose (PD groups) administered twice daily. Afterwards, animals were sacrificed, and samples from visceral as well as parietal peritoneum were obtained. The samples were stained with Sirius-Red, and at 10 different sites per sample, peritoneal membrane thickness was measured using computer-aided histomorphometry devices.

Results. Mean peritoneal membrane thickness was increased by PD in both WT and ETB⁻/⁻ rats versus respective Sham controls (WT Sham: 22.3 ± 0.7 µm/ETB Sham: 22.3 ± 0.9 µm versus WT PD: 26.5 ± 1.5 µm/ETB PD: 28.7 ± 1.2 µm; P < 0.05, respectively). However, no difference in peritoneal membrane thickness was detected between WT PD and ETB⁻/⁻ PD groups.

Conclusion. Our study demonstrates that PD increases peritoneal membrane thickness in a rat model, but deficiency of the ETB receptor has no detectable impact on this process.

Keywords: endothelin; ETB receptor; peritoneal membrane thickening

Introduction

Peritoneal fibrosis is a common complication in patients with end-stage renal disease on peritoneal dialysis (PD) [1]. The pathophysiological mechanisms involved in the process are only partially known. However, endothelin-1 (ET-1) is a potent pro-inflammatory and pro-fibrotic mediator, as its major biological effects include the induction of mitogenesis of fibroblasts, smooth muscle cells and myocytes, activation of neutrophils and the induction of fibronectin as well as chemotaxis of fibroblasts [2]. It acts via two receptors: the endothelin A (ETA) and endothelin B (ETB) receptor [3,4].

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