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Original article

Involvement of the NF-κB/p50/Bcl-3 complex in response to antiangiogenic therapy in a mouse model of metastatic renal cell carcinoma

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ABSTRACT

Renal cell carcinoma (RCC) represents approximately 2–3% of human malignancies. Nuclear transcription factor κB (NF-κB) is composed of a family of transcription factors that have been associated with the development and progression of RCC. Endostatin (ES) is a fragment of collagen XVIII that possesses antiangiogenic activity. In this study, we evaluated the expression of NF-κB in metastatic tumor cells from animals treated with ES. Balb/c-bearing Renca-EGFP cells were treated with NIH/3T3-LendSN or NIH/3T3-LXSN cells as a control. At the end of the in vivo experiment, plasma Renca-EGFP-sorted cells and tissue lung samples were collected. A real-time PCR array for NF-κB target genes revealed that ES therapy led to down regulation of Bcl-3 ($P < 0.031$), NF-κB1 ($P < 0.001$) and c-Rel ($P < 0.004$) in the ES-treated group. Using an electrophoretic mobility shift assay (EMSA), we observed a reduction in NF-kB binding activity in ES-treated Renca-EGP cells. Furthermore, a supershift assay showed a clear shift of the NF-κB DNA band in samples incubated with a p50 antibody. By immunohistochemistry analysis, ES treatment resulted in a significant reduction in expression of p50. (ES vs. control $P < 0.05$). The immunoprecipitation experiments confirmed the presence of a p50/Bcl-3 complex in nuclear extracts from cells of metastatic lung tissues. Our findings indicate that p50 and Bcl-3 plays a regulatory role in gene transcription in RCC.

1. Introduction

Renal cell carcinoma (RCC) is the most common renal cancer and accounts for 90–95% of kidney neoplasms [1]. This malignancy is clinically and pathologically heterogeneous, and clear cell renal carcinoma (ccRCC) the most common type in adults. Most cases of primary RCC are treated with partial or radical nephrectomy. However, the incidence of metastatic disease following surgery is approximately 40% [2]. The high incidence of metastatic disease has been associated with a RCC angiogenic phenotype, which results from a mutation or hypermethylation of the von Hippel-Lindau (VHL) gene, with subsequent hypoxia-inducible factor (HIF) activation. Overexpression of the HIF protein results in increased expression of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), which are key players involved in ccRCC development and progression [3,4].

One of the molecules involved in the development and progression of RCC is the nuclear transcription factor κB (NF-κB). NF-κB is a pleiotropic transcription factor belonging to the Rel/NF-κB family that participates in the activation of many genes, including cytokines and metalloproteinases (MMPs) [5]. In mammals there are five known members of the Rel family: RelA (p65), RelB, c-Rel, NF-κB1 105/p50, and NF-κB2-p100/p52. NF-κB1-p105/p50 and NF-κB2-p100/p52 are precursor molecules that undergo cleavage of the N-terminal region in the proteasome, generating the mature proteins p50 and p52. These subunits homo or heterodimerize to form activator dimers (p50/p65) and repressors (p50/p50 and p52/p52) [6,7]. NF-κB dimers bind at a consensus sequence (IkB sites, 5′GGRRNNYYCC3′, R = purine Y = pyrimidine). Without stimulation, NF-κB remains in the

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cytoplasm in its inactive form linked to one of its inhibitors (IKBs), including IkB-α, IkB-β, IkB–ε, IkBγ, IkBδ and Bcl-3 [6].

The best-described NF-κB pathways are classical and alternative pathways or canonical and non-canonical pathways, respectively [7,8]. The canonical pathway is activated by inflammatory cytokines and viral infections. Upon activation of this pathway, IKB is phosphorylated, ubiquinated and hence degraded by the proteasome. Free NF-κB (p65 and p50) migrates to the nucleus where it activates transcription of specific genes. The non-canonical pathway is activated by members of the tumor necrosis factor (TNF) family, such as the B cell activating factor (BAFF) lymphotoxin B. In this pathway, IKKα is activated and subsequently phosphorylates p100. The p100 protein is ubiquinated and processed, yielding mature p52. Thus, the RelB/p52 heterodimer migrates to the nucleus where it activates transcription of specific genes [5,9]. According to Gilmore, other distinct pathways must exist. For example, the p50 homodimer together with Bcl-3 can act as a transcriptional activator [7]. Furthermore, an association between the NF-κB activator (the Bcl-3/p50 complex) and development of nasopharyngeal carcinoma has been shown [10].

Over the last decade, papers have correlated NF-κB activation with angiogenesis, chemotherapy resistance and apoptosis resistance in RCC. Oya et al. were the first to demonstrate constitutive activation of NF-κB in RCC cell lines using an electrophoresis mobility shift assay (EMSA). Moreover, high NF-κB activity was inversely correlated with TRAIL-induced apoptosis. Supershift assay results showed that p50 was involved [11]. The same group also studied NF-κB expression in RCC tissue samples and found a positive correlation between the histological grade, invasion, metastasis and inflammatory paraneoplastic syndrome by activating NF-κB [12].

Two independent groups, Meteoglu et al. and Dordevic et al., analyzed histological samples of patients with metastatic RCC and found that the increase in NF-κB (p50) activity correlated with increased angiogenic and apoptotic markers, such as epidermal growth factor receptor (EGFR), VEGF, bcl-2 and p53 [9,13]. An et al. studied the effect of NF-κB blockade with an IkB super repressor, alone and with a proteasome inhibitor (bortezomib), to induce apoptosis in RCC cells. The authors found that specific inhibition of NF-κB was not sufficient to induce apoptosis. However, blocking NF-κB was necessary to induce apoptosis in RCC cells with bortezomib [14]. The impact of inhibiting NF-κB activation by pyrrolidine dithiocarbamate (PDTC) in RCC was evaluated by Morais et al. [1,15,16]. Both, *in vitro* and *in vivo* studies showed that PDTC played a role in inhibition of NF-κB expression and consequently decreased viability and proliferation in RCC.

Endostatin (ES) was originally isolated from hemangioendothelioma and identified as the carboxy-terminal segment of collagen XVIII [17]. ES has become a focus of medical interest because of its tumor angiogenic activity [18]. In 2004, Abdollahi et al. analyzed gene expression in human endothelial cells treated with ES and found that NF-κB was one of the factors influenced by ES in its inhibition of angiogenesis [19].

In recent years, our group has been working with retroviral gene therapy using ES for the treatment of primary and metastatic RCC in animal models. ES showed antiangiogenic and antitumor activities. Furthermore, it was shown that ES caused exhibits potent immunomodulatory effects [20–26].

In this study, we investigated the regulation of NF-κB activity and metastatic Renca-EGFP after ES treatment.

2. Methods

2.1. Cell lines

NIH/3T3-LendSN-clone 3 was utilized for ES expression, and NIH/3T3-LXSN was used as a control, as described in previous work [23,26]. Both cell lines were maintained in high-glucose (4.5 g/L at 25 mM) DMEM medium (Life Technologies Corporation®, Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco®), and 10% fetal bovine serum (FBS). The murine kidney carcinoma cell lines (Renca), were purchased from CLS Cell Lines Service® (Eppelheim, Germany). The cells were transfected with enhanced green protein fluorescent (EGFP) maintained in RPMI 1640 medium (Life Technologies Corporation®, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% minimal Eagle's medium nonessential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco®). All the cell lines were maintained in a humidified chamber at 37 °C and under 5% carbon dioxide.

2.2. Animals

Male Balb/c mice (age 8–10 weeks) were obtained from the Animal Facility of IPEN/CNEN-SP, Sao Paulo, Brazil. In these experiments, 35 mice were used (normal = 5, control group = 15, ES-treated group = 15). All animals were cared for in accordance with the standards of the institute under a protocol approved by the Animal Experimentation Ethics Committee (Number of Process: 87/11).

2.3. Orthotopic RCC tumor model

Mice were anesthetized by an intraperitoneal subcutaneous injection of a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively). The left kidney was exposed through a left flank incision and was partially exteriorized. Using a Hamilton syringe with a 27-gauge needle, 2×10^5 Renca-EGFP cells in 10 μL of phosphate buffered saline (PBS) were injected under the renal capsule. Then, the kidney was then allowed to fall back into the abdominal cavity. The body wall and the skin incision were closed separately with absorbable 5-0 vicryl sutures. The left kidney was removed by unilateral nephrectomy 7 days after inoculation with the Renca-EGFP cells. The mice were divided into two experimental groups: the control group and the ES-treated group. Each group received a subcutaneous injection of 3.6×10^6 NIH/3T3-LXSN or NIH/3T3-LendSN-clone 3.

2.4. ELISA analysis

At the end of the experiment, blood samples from the mice were taken into test tubes with heparin. Then, the plasma components were separated and frozen at −20 °C. Samples were thawed at the time of the study, and plasma ES levels and NIH/3T3-LendSN-clone 5 levels were measured using a Mouse Endostatin ELISA Kit (Novateinbio® – Accelerates Research and Development – USA) according to the manufacturer's instructions. The ES concentrations were determined at least in duplicate, and the assay reproducibility was confirmed. ELISA plates were read using the Multiskan EX Microplate Reader (Labsystems, Milford, MA, USA).

2.5. Sorting

At the end of the experiment, the metastatic lung tissues were digested with a solution of RPMI and Collagenase A (Roche, Mannheim, Germany) (3:1) to obtain a cell homogenate, which was then submitted to analysis by flow cytometry (BD FACSAria II). We adjusted the size and fluorescence intensity of the red and blue lasers. The initial parameter settings for cell analysis were defined as the "sorting" strategy using the FITC+ (green) cells. These parameters also provided for a homogeneous size distribution