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# Pro-inflammatory cytokine and high doses of ionizing radiation have similar effects on the expression of NF-kappaB-dependent genes

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ABSTRACT

The NF-κB transcription factors are activated via diverse molecular mechanisms in response to various types of stimuli. A plethora of functions associated with specific sets of target genes could be regulated differentially by this factor, affecting cellular response to stress including an anticancer treatment. Here we aimed to compare subsets of NF-κB-dependent genes induced in cells stimulated with a pro-inflammatory cytokine and in cells damaged by a high dose of ionizing radiation (4 and 10 Gy). The RelA-containing NF-κB species were activated by the canonical TNFα-induced and the atypical radiation-induced pathways in human osteosarcoma cells. NF-κB-dependent genes were identified using the gene expression profiling (by RNA-Seq) in cells with down-regulated *RELA* combined with the global profiling of RelA binding sites (by ChIP-Seq), with subsequent validation of selected candidates by quantitative PCR. There were 37 NF-κB-dependent protein-coding genes identified: in all cases RelA bound in their regulatory regions upon activation while downregulation of *RELA* suppressed their stimulus-induced upregulation, which apparently indicated the positive regulation mode. This set of genes included a few "novel" NF-κB-dependent species. Moreover, the evidence for possible negative regulation of *ATF3* gene by NF-κB was collected. The kinetics of the NF-κB activation was slower in cells exposed to radiation than in cytokine-stimulated ones. However, subsets of NF-κB-dependent genes upregulated by both types of stimuli were essentially the same. Hence, one should expect that similar cellular processes resulting from activation of the NF-κB pathway could be induced in cells responding to pro-inflammatory cytokines and in cells where so-called "sterile inflammation" response was initiated by radiation-induced damage.

## 1. Introduction

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factor is a protein complex regulating cell response to different types of stimuli. Its primary function is regulation of immune response and inflammation (e.g. expression of cytokines). Other genes, which transcription depends on the NF-κB, include those coding for proteins involved in apoptosis, activation of cell cycle progression, angiogenesis, and metastasis. In general, the κB responsive element could be found in regulatory regions of several hundred genes [1]. Moreover, up-regulation of the NF-κB pathway is frequently observed in cancer cells, which may contribute to their resistance to the anticancer treatment [2,3].

NF-κB transcription factors are dimers formed by members of the multigene NFκB/Rel family, which in humans include five proteins: RelA(p65), RelB, c-Rel, NF-κB1(p50), and NF-κB2(p52); latter two proteins are synthesized as larger precursors p105 and p100, respectively. NF-κB subunits require heterodimerization or homodimerization for transcriptional activity; homodimers of p50 or p52 can lead to inhibition of gene expression in certain cases [4]. Generally, in resting

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cells, NF-κB dimers are sequestered in the cytoplasm by association with inhibitory proteins called IκB, which prevents binding of NF-κB to DNA. Expression of IκB (the major inhibitor of NF-κB) is controlled by an NF-κB-responsive promoter, which together with activation of other NF-κB-dependent target *TNFAIP3* gene encoding for A20 protein generates the major internal circuits of autoregulation of NF-κB signaling. Pro-inflammatory extracellular signals or cellular stress can induce activation of IKK kinase, which in turn phosphorylates IκB protein. The core IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO/IKKγ. Phosphorylation of IκB causes its ubiquitination and degradation in the proteasome, which allows the translocation of NF-κB to the nucleus and its binding to the κB DNA regulatory elements [5].

Depending on the stimulation, cell type, and cellular context, NF-κB can be activated through different mechanisms [6], yet all of them lead to freeing the NF-κB from its inactive complex, translocation to the nucleus and binding to the promoter and enhancer regions of regulated genes. The RelA(p65)/NF-κB1(p50) heterodimer is the most abundant NF-κB form, which is involved in so-called “classical” or “canonical” NF-κB pathway. This classical NF-κB pathway, the major regulator of the inflammatory response, is primarily activated by pro-inflammatory stimulation, among others by the TNFα cytokine, and involves IKKβ-catalyzed phosphorylation and subsequent proteolysis of inhibitory protein IκBα [7]. Moreover, the alternative (“non-canonical”) pathway exists, which involves the IKKα-mediated phosphorylation and processing of p100, leading to induction of p52-containing NF-κB complexes [8,9]. Additionally, a number of “atypical” pathways have also been described, including radiation inducible mechanisms of NF-κB activation [10–12]. DNA double-strand break, the critical form of damage induced by ionizing radiation (IR), can activate the NF-κB signaling [13,14]. This mechanism involves multiple pathways of ATM-mediated activation of IKK via NEMO/IKKγ, which leads to phosphorylation and proteolytic degradation of IκBα [15–18]. Moreover, it has been also reported that ATM kinase could directly phosphorylate RelA(p65) at Ser-547, which modulates expression of a subset of NF-κB-dependent genes [19].

Although IR-induced ATM-mediated pathway of NF-κB activation can be called atypical, several lines of evidence indicate that the major effector of this pathway is RelA(p65)/NF-κB1(p50) heterodimer [11,19]. Considering the common downstream effector of an inflammation-related classical NF-κB pathway and IR-induced atypical NF-κB pathway one might expect similar subsets of NF-κB-dependent genes activated in both circumstances. We aimed here to compare directly subsets of NF-κB-activated genes in cells stimulated with TNFα cytokine, an established trigger of classical NF-κB pathway, and in cells exposed to a high dose of IR. To confer dependency of induced NF-κB pathway on RelA(p65)/NF-κB1(p50) heterodimer, stimulus-induced promoter binding of RelA(p65) was addressed and gene expression was analyzed also in cells with RelA(p65) downregulated by siRNA.

## 2. Methods

### 2.1. Experimental model

Human osteosarcoma U-2 OS cells were selected as an experimental model where activation of the classical NF-κB pathway and p53-dependent response to ionizing radiation was already extensively studied [20–22]. The wild-type U2-OS cells were purchased from ATCC (HTB-96) and used as a primary model. Additionally, cells were transiently transfected with 10 nM siRNA (Eurofins) using INTERFERin® Kit (Polyplus Transfection) for 48 h with sequences specific for *RELA* gene (5′-GCUGAUGUGCACCGACAAG-3′; upper strands) or control siRNA (5′-CAGUCGCGUUUGCGACUGG-3′). Cells were grown in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and gentamicin (40 mg/ml; Krka) at 37 °C in a humidified 5% $CO_2$ atmosphere.

### 2.2. Cell treatments

Any treatment started 48 h after inoculation of cells; all steps, with the exception of irradiation, were carried out at 37 °C and 5% $CO_2$. For gene expression analysis cells were incubated with TNFα cytokine (10 ng/ml) (T0157; Sigma) and harvested after 0.5 or 4 h of stimulation. Alternatively, cells were irradiated with 4 or 10 Gy IR dose at a 1 Gy/min dose rate using 6 MeV photons (generated by linear accelerator Clinac 600, Varian), the medium was replaced with a fresh one, and cells were analyzed 4 h after irradiation. For ChIP-Seq analysis *wild-type* cells were harvested after 0.5 and 4 h of stimulation with TNFα (30 ng/ml), or 2 and 4 h after irradiation at 10 Gy.

### 2.3. Immunocytochemistry

At appropriate experimental time points cells were washed three times with PBS and fixed with 4% formaldehyde solution in PBS for 20 min. After three washes with PBS, cells were permeabilized in 0,1% Triton X-100/0.1 × citrate buffer for 5 min on ice and washed three times with PBS. Non-specific binding of antibody was blocked with a 3% BSA solution in PBS for 30 min at room temperature. The preparations were incubated with the anti-RelA(p65) antibody (ab7970; Abcam) in 3% BSA/PBS for 1 h, then with secondary Ab conjugated with FITC (2% BSA/PBS) for 1 h in dark. Cell nuclei were counterstained with DAPI solution and washed. The preparations were examined with a confocal microscope at 40 × magnification.

### 2.4. Western blot analysis

Whole-cell lysates were obtained from cells treated with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) supplemented with a Complete™ protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). To isolate nuclei, cells were incubated for 10 min in an ice-cold lysis buffer (20 mM Tris pH 7.6, 10 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 0.5% NP40, 0.25 M sucrose, and protease inhibitor cocktail), centrifuged at 400*g* for 10 min, and resulting pellet (nuclei) was washed twice with PBS by centrifugation. Isolated nuclei were incubated in extraction buffer (20 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.4 M NaCl, 25% glycerol, and protease inhibitor cocktail) for 30 min at 4 °C with gentle shaking. Equal amounts of proteins (25 μg; estimated using the Bradford assay, Biorad) were separated using 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore). The membranes were blocked with 5% nonfat milk in Tris-buffered saline and then incubated overnight at 4 °C with antibodies specific for human RelA(p65) (C20; Santa Cruz), ATF3 (#33593; Cell Signaling) or actin (#4967; Cell Signaling). Proteins were visualized after incubation with a peroxidase-conjugated secondary antibody using the enhanced chemo-luminescence kit (Pierce) according to the manufacturer's instructions.

### 2.5. Global gene expression profiling

Total RNA was extracted from $1 \times 10^6$ cells using RNeasy Mini Kit (Qiagen) and treated on-column with DNase using the RNase-Free DNase Set (Qiagen). Preparation cDNA libraries and sequencing by Illumina HighSeq 2500 (run type: single read, read length: 1 × 50 bp) were carried out by GATC Biotech AG, Germany (www.gatc-biotech.com). Raw RNA-Seq reads were aligned to human genome hg19 using tophat2 [23] with Ensembl genes transcriptome reference. Aligned files were processed using Samtools [24]. Furthermore, reads aligned in the coding regions of the genome were counted using FeatureCounts [25]. Finally, read counts were normalized using DESeq2 [26], then normalized expression values were subject to differential analysis (mean based fold change) and statistical testing using the Student *t*-test in the R/Bioconductor programming environment. In general, transcripts of 25,369 genes were detected, yet genes with very low signals were