

## Cytokine action and oxidative stress response in differentiated neuroblastoma SH-SY5Y cells<sup>★</sup>

Joanna Kania, Aleksandra Barańska and Amalia Guzdek<sup>✉</sup>

*Department of Cell Biochemistry, Faculty of Biotechnology, Jagiellonian University, Kraków, Poland*

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**In the retinoic acid-differentiated neuroblastoma SH-SY5Y cells, IL-1 induced binding activity of NF $\kappa$ B and up-regulated the expression and activity of MnSOD. The IL-1-elicited effects were partly reversed by IL-4 and IL-6. It is proposed that IL-4 and IL-6 may participate in the regulation of the imbalanced oxidant status induced by IL-1 in differentiated neuroblastoma cells. In the SH-SY5Y cell line, TNF $\alpha$  neither activated NF $\kappa$ B nor induced MnSOD expression and activity, but was capable of modulating the IL-1 effects. Pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF $\kappa$ B activation, down-regulated the expression and activity of MnSOD, which may suggest that the regulation of MnSOD by IL-1 in retinoic acid-differentiated neuroblastoma cells was mediated by the nuclear factor  $\kappa$ B.**

The oxidative stress-responsive nuclear factor  $\kappa$ B (NF $\kappa$ B) is the main regulator of immune-related functions (Pahl, 1999; Bowie & O'Neil, 2000). By binding to specific sites in the promoter region, it regulates a number of genes associated with inflammation, lymphoid organ development, cell adhesion and apoptosis (Karin & Lin, 2002). In several

types of cells, activation of NF $\kappa$ B is central to the regulation of many genes by proinflammatory cytokines such as IL-1 $\beta$  or TNF $\alpha$  which rapidly induce NF $\kappa$ B DNA-binding activity. The activation pathway utilizes the IKK $\beta$  catalytic subunit, and is accomplished by I $\kappa$ B phosphorylation, followed by ubiquitin-dependent degradation (Israel,

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<sup>✉</sup>To whom correspondence should be addressed: Amalia Guzdek, Department of Cell Biochemistry, Faculty of Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland; tel.: (48 12) 252 6341; fax: (48 12) 252 6902; e-mail: Amag@mol.uj.edu.pl

**Abbreviations:** BCA, bicinchoninic acid; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; NF $\kappa$ B, nuclear factor kappa B; NBT, nitro blue tetrazolium; MnSOD, manganese superoxide dismutase; PDTC, pyrrolidine dithiocarbamate; RA, retinoic acid.

2000). It has been well established that oxidative stress can trigger the production of cytokines which, in turn, induce the synthesis of proteins such as manganese superoxide dismutase (MnSOD) (Antras-Ferry *et al.*, 1997; Rogers *et al.*, 2001), which protects cells against the damage caused by reactive oxygen species. Xu *et al.* (1999) cloned human MnSOD gene, and found several NF $\kappa$ B binding sites in the 3' and 5' flanking and the intronic regions. The authors suggested that the location of NF $\kappa$ B elements in the MnSOD gene is critical for the IL-1-elicited induction, but in cooperation with other transcription elements. In the present study we examined the effects of IL-1 $\beta$  and/or IL-4, IL-6 and TNF $\alpha$  on the activation of NF $\kappa$ B and on the activity and expression of MnSOD in differentiated neuroblastoma cells. A number of neurodegenerative disorders are characterised by an increase in the level of proinflammatory cytokines (Neuroinflammatory Working Group, 2000). These cytokines can modulate several intracellular signal transduction pathways in neuronal cells, e.g. *via* activation of NF $\kappa$ B or induction of COX-2 expression (Fiebich *et al.*, 2000), which may lead to cell destruction. Neuroblastoma cell culture appears to be a good experimental model for examining the influence of cytokine network on neuronal metabolism. SH-SY5Y cells can be morphologically differentiated into neuronal cells, whose phenotype varies depending on the inducing factors, e.g. retinoic acid, nerve growth factor, dibutyryl cyclic AMP or TPA (Yu *et al.*, 1988).

## MATERIALS AND METHODS

SH-SY5Y cells were kindly donated by Prof. A. Szczudlik (Collegium Medicum, Jagiellonian University, Kraków, Poland). IL-6 was provided by Prof. P.C. Heinrich (Germany), and IL-1 $\beta$  by Prof. Ch. Dinarello (U.S.A.). The DMEM medium and fetal bovine serum (FBS) were purchased from Gibco Life Technologies

Inc. (U.S.A.). [ $\alpha$ <sup>32</sup>P]dCTP was from ICN Pharmaceuticals, Inc. (U.S.A.), and the MnSOD cDNA probe from the ATCC (U.S.A.). Antibiotics (penicillin, streptomycin) were obtained from Polfa (Tarchomin, Poland), human IL-4 and other reagents from Sigma.

**Cell culture.** SH-SY5Y neuroblastoma cells were cultured at 37°C in 75 cm<sup>2</sup> flasks containing DMEM supplemented with 10% FBS and antibiotics under a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The cells were differentiated with retinoic acid (RA) (10  $\mu$ M) for 72 h (Scheibe & Wagner, 1992). For isolation of nuclear proteins and total RNA, as well as for MnSOD activity assay, the SH-SY5Y cells were cultured in 60 mm Petri dishes. The medium was changed 24 h before addition of the following cytokines: IL-1 $\beta$  (10 ng/ml) and/or TNF $\alpha$  (10 ng/ml), IL-4 (100 U/ml) and IL-6 (25 ng/ml). In some assays, the culture medium was supplemented with pyrrolidine dithiocarbamate (PDTC) (100  $\mu$ M) 1 h prior to IL-1 addition.

Nuclear extracts were isolated after 90 min, and total cellular RNA after 8 h of cytokine or PDTC treatment. The activity of MnSOD was estimated in cells cultured with the specific cytokines or PDTC for 24 h.

**Nuclear protein extraction and EMSA.** Nuclear extracts were prepared by a mini-extraction procedure (Suzuki, 1994). The retinoic acid-differentiated neuroblastoma cells were cultured for 90 min with IL-1 (10 ng/ml), or a mixture of IL-1 and TNF (10 ng/ml), or IL-4 (100 U/ml), or IL-6 (25 ng/ml); they were then washed with cold phosphate-buffered saline (PBS), collected and centrifuged for 5 min at 400  $\times$  g. The cells, previously resuspended in a buffer (10 mM NaCl, 3 mM MgCl<sub>2</sub>, 10 mM Tris, pH 7.5, and 0.2 mM phenylmethylsulfonyl fluoride, PMSF), were incubated on ice for 15 min. Nonidet NP-40 was added, and samples were centrifuged for 60 s at 14 000 r.p.m. Pelleted nuclei were resuspended in a buffer (10 mM Hepes, 0.35 M NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT) and 0.2 mM PMSF) and centrifuged for 5 min

at 14 000 r.p.m. After centrifugation at 4°C, the supernatant proteins were measured by the BCA method. The remainder of the supernatant was frozen in 10% glycerol.

For NFκB activity assay (a DNA electrophoretic mobility shift assay), nuclear protein extracts (10 μg) were incubated for 30 min at a room temperature in 25 μl of the binding buffer (0.5% Triton X-100, 2.5% glycerol, 10 mM Hepes, 4 mM DTT) containing 0.5 ng of <sup>32</sup>P-end-labelled NFκB-binding oligonucleotide (about 10<sup>5</sup> c.p.m.) and 1 μg of poly(dI-dC) (which was used as competitor). DNA-protein complexes were separated in a 5% polyacrylamide gel for 1.5 h at 140 V. The dried gels were analysed by autoradiography. The relative intensity of the bands was evaluated densitometrically using the computer imaging system Fluor S MultiImager (BioRad).

**RNA extraction and Northern blots.** The Chomczynski's extraction method (Chomczynski & Sacchi, 1987) and isopropanol precipitation were used for total RNA isolation from the neuroblastoma cells treated for 8 h with the analysed cytokines or PDTC. RNA samples (10 μg) were separated by electrophoresis in a 1% agarose gel under denaturing conditions. After electrophoresis, RNA was transferred to Hybond-N membranes (Amer-

sham) according to the manufacturer's instructions. The filters were prehybridized at 68°C for 3 h in 10% dextrane sulphate and 1% SDS, and were hybridized in the same solution overnight at 65°C with a <sup>32</sup>P-labelled human MnSOD cDNA probe and subjected to autoradiography. The relative intensity of the bands was evaluated densitometrically using the computer imaging system Fluor S MultiImager (BioRad).

**SOD activity evaluation.** The cells were treated with cytokines or PDTC for 24 h, washed twice with cold PBS, harvested in 0.2 ml of PBS, frozen and thawed four times in liquid nitrogen, each time under stirring. The mixture was centrifuged for 2 min (14 000 r.p.m., 4°C) and the supernatant was used for SOD activity measurement. A BCA kit was used for cellular protein estimation. Protein samples (10 μg) were separated in a 15% polyacrylamide gel, 180 V, for 80 min in a Tris/glycine buffer. After electrophoresis, the gel was immersed in a staining buffer containing riboflavin (50 mM phosphate buffer, pH 7.8, 10 mM EDTA, 245 μM nitro blue tetrazolium, NBT, 28 mM TEMED, 30 μM riboflavin), stirred in the dark and then exposed to light until white bands appeared on a blue background.

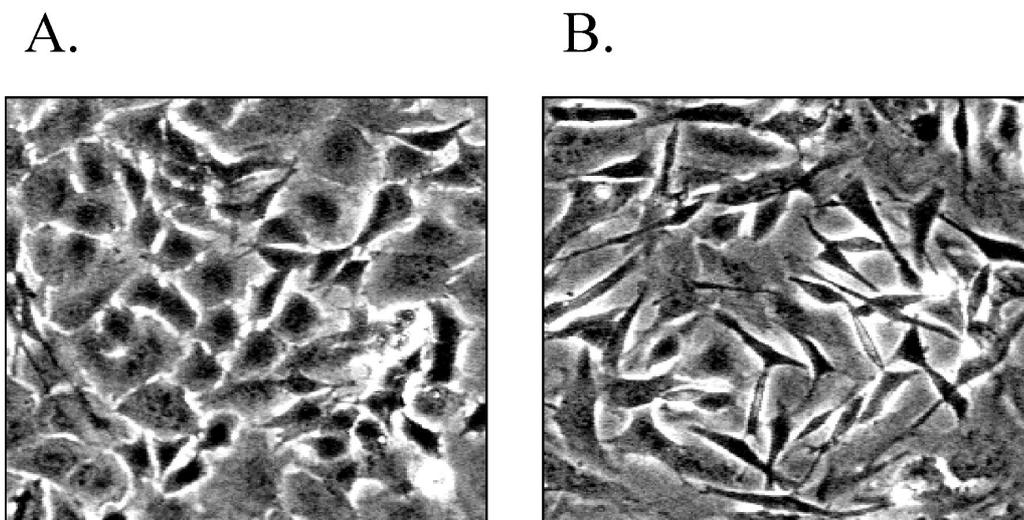


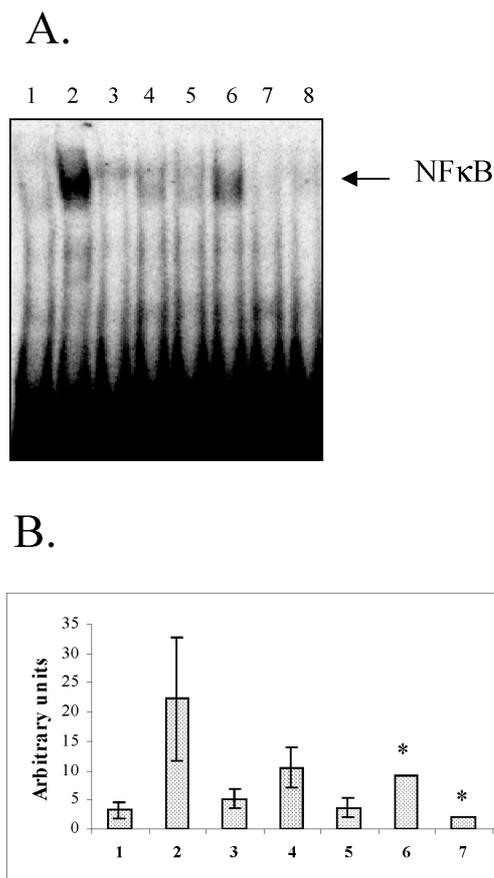
Figure 1. Morphological changes in SH-SY5Y human neuroblastoma cells after treatment with 10 μM retinoic acid for 72 h.

A, control; B, differentiated cells.

## RESULTS

As shown in Fig. 1 exposure of SH-SY5Y cells to 10  $\mu$ M RA for 72 h led to their differentiation. We used a NF $\kappa$ B consensus oligonucleotide for EMSA analysis to determine whether IL-4, IL-6 or TNF cooperate with IL-1 in NF $\kappa$ B activation. Figure 2 demonstrates that IL-1 is the main inducer of this transcrip-

greatly enhanced by IL-1, but the modifying effect exerted by IL-4, IL-6 and TNF was less potent (Fig. 4). It was found previously that retinoic acid affected MnSOD protein stability, but not expression (Ahlemeyer *et al.*, 2001), hence some interference of the RA-produced effects with those of IL-4, IL-6 or TNF should be considered. Neither of the cytokines examined was capable of affecting CuZnSOD activ-



**Figure 2. Effect of treatment (for 90 min) with IL-1 (10 ng/ml) and/or IL-4 (100 U/ml), IL-6 (25 ng/ml), TNF (10 ng/ml) on NF $\kappa$ B activation.**

The cell extract was incubated with a radioactively labelled oligonucleotide containing a NF $\kappa$ B consensus binding sequence. The reaction mixture was separated on a native polyacrylamide gel. Lane: 1, control; 2, IL-1; 3, IL-4; 4, IL-1+IL-4; 5, IL-6; 6, IL-1+IL-6; 7, TNF; 8, IL-1+TNF. **A.** Representative gel; **B.** Scanning data presented in arbitrary units; mean of three experiments. The bars represent  $\pm$  S.D. mean of two results.

tion factor. In contrast, TNF $\alpha$  had no effect on NF $\kappa$ B binding activity, and even decreased the IL-1-elicited NF $\kappa$ B activation. However, these results should be further analysed. As expected IL-4 and IL-6 decreased the NF $\kappa$ B activation induced by IL-1.

The regulation of MnSOD expression by the tested cytokines showed a response pattern similar to that observed for NF $\kappa$ B activation (Fig. 3). IL-1 distinctly up-regulated MnSOD expression in the differentiated neuroblastoma cells, whereas IL-4, IL-6 and TNF partly reversed that effect. The enzyme activity was

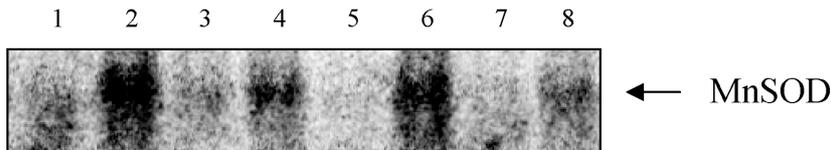
ity (Fig. 4), but PDTC, an inhibitor of NF $\kappa$ B activation (Bowie *et al.*, 1997), diminished the activity of this enzyme (Fig. 5B). PDTC inhibited both the IL-1-induced NF $\kappa$ B binding (Fig. 6) and the IL-1-elicited MnSOD expression and activity (Fig. 5A, B).

## DISCUSSION

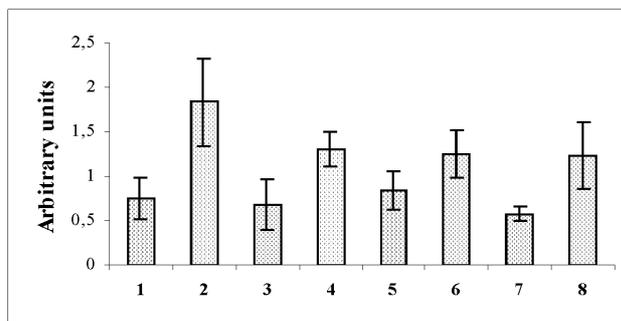
In the present report we describe the effects of proinflammatory (IL-1, TNF) and so-called antiinflammatory cytokines (IL-4, IL-6) on

the oxidant status of RA-differentiated (as demonstrated by morphological criteria) neuroblastoma cells, as measured by activation of the oxidative stress-responsive NF $\kappa$ B, as well as by expression and activity of the main inducible antioxidant enzyme MnSOD.

A.



B.

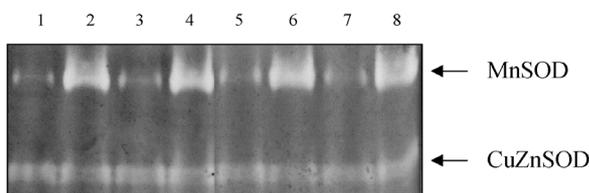


**Figure 3. Effect of IL-1 (10 ng/ml) and/or IL-4 (100 U/ml), IL-6 (25 ng/ml), TNF (10 ng/ml) on MnSOD mRNA expression.**

Total RNA was analysed by Northern blot hybridization. 1, control; 2, IL-1; 3, IL-4; 4, IL-1+IL-4; 5, IL-6; 6, IL-1+IL-6; 7, TNF; 8, IL-1+TNF.

**A.** Representative blot; **B.** Scanning data presented in arbitrary units after normalization against 18 sRNA; mean of three experiments; the bars represent  $\pm$ S.D.

Several studies have reported the induction of NF $\kappa$ B binding activity by IL-1 (Fig. 2), whereas the effectiveness of TNF seems to be cell-type-specific. As is shown in Fig. 2, TNF is unable to activate NF $\kappa$ B in the RA-differentiated neuronal SH-SY5Y cells. A similar observation was made by Wong (1995) with

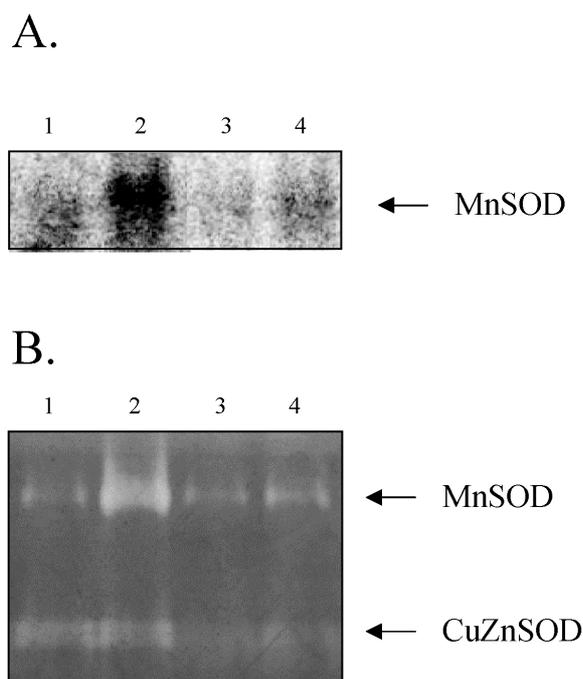


**Figure 4. Influence of IL-1 (10 ng/ml) and/or IL-4 (100 U/ml), IL-6 (25 ng/ml), TNF (10 ng/ml) on the activity of MnSOD and CuZnSOD in cells cultured for 24 h with the indicated cytokines.**

1, control; 2, IL-1; 3, IL-4; 4, IL-1+IL-4; 5, IL-6; 6, IL-1+IL-6; 7, TNF; 8, IL-1+TNF.

neuroblastoma SK-N-SH cells and some other tumour cells. The nuclear factor  $\kappa$ B is regarded as an antiapoptotic factor in neuroblastoma cells (Yabe *et al.*, 2001; Bian *et al.*, 2002). However, prolonged activation of NF $\kappa$ B may be dangerous to the cell. It seems

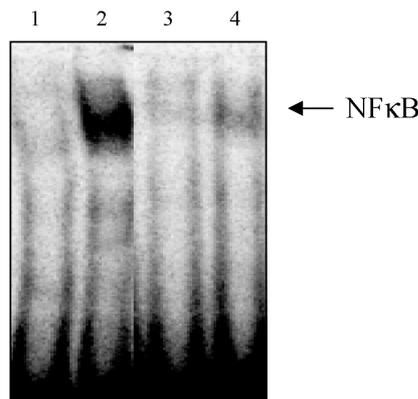
that IL-4 and IL-6 can abrogate the IL-1-elicited effect on nuclear factor  $\kappa$ B activation. Cellular responses to cytokines depend on receptors, signalling molecules and the stage of cell differentiation. The cytokine-activated transcription factors NF $\kappa$ B, C/EBP and STATs regulate individually or cooperatively the expression of target genes. Gene expression is determined by interactions between transcription factors, the promoter context of the target gene, and the presence of co-activator complexes. A cross-talk between the C/EBP, STAT and NF $\kappa$ B signal transduction pathways has been postulated (Luo & Yu-Lee, 2000; Kiningham *et al.*, 2001; Cisowski *et al.*, 2002). It is possible that the STAT pathway, which is activated by IL-4 or IL-6, may inhibit the NF $\kappa$ B binding induced by IL-1 in neuroblastoma cells (experiments in progress).



**Figure 5.** Effect of PDTC (100  $\mu$ M) on MnSOD expression (A) and MnSOD and CuZnSOD activity (B).

Lane 1, control; 2, IL-1; 3, PDTC; 4, IL-1+ PDTC.

The brain is particularly susceptible to oxygen free radicals which are implicated in the pathology of several neurological disorders (Jenner, 2003; Pong, 2003; Klein & Ackerman, 2003). The antioxidant enzyme system of the brain may play an important role in the protection against oxidative stress. The induction of MnSOD, an enzyme converting superoxide anion to hydrogen peroxide, by IL-1 can protect cells against the damaging effects of reactive oxygen species. MnSOD overexpression, in human neuronal cells expressing mutant CuZnSOD, attenuated neuronal death (Flanagan *et al.*, 2002). CuZnSOD – overexpressing astrocytoma cells also show increased resistance to oxidative injury (Chen *et al.*, 2001). IL-4 and IL-6 seem to be capable of normalizing the IL-1-produced effects. Depending on the activation of astrocytes, glial and neuronal cells in the CNS, different subsets of cytokines can be generated; they can modulate neuronal cell metabolism resulting in cell



**Figure 6.** Inhibitory effect of PDTC (100  $\mu$ M) on NF $\kappa$ B activation in neuroblastoma cells treated with IL-1 (10 ng/ml) for 90 min.

Lane 1, control; 2, IL-1; 3, PDTC; 4, IL-1+PDTC.

protection or damage (Klegeris & McGeere, 2001).

Pyrollidine dithiocarbamate (PDTC), a metal chelator and antioxidant, inhibits NF $\kappa$ B due to its dithiocarbamate moiety (Bowie *et al.*, 1997). Dithiocarbamates have been used clinically for treating various pathogenic fungi and bacteria, as well as in agricultural insecticides, herbicides and fungicides (Nobel *et al.*, 1995). PDTC inhibits CuZnSOD activity (Erlejman & Oteiza, 2002; and Fig. 5B) and – as was shown in our experiments – decreases the IL-1 induced MnSOD activation. On the basis of the above observations we postulate that the transcription factor  $\kappa$ B is crucial for MnSOD up-regulation in differentiated neuroblastoma cells.

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