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Tec kinase stimulates cell survival in transfected Hek293T cells and is regulated by the anti-apoptotic growth factor IGF-I in human neutrophils

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ABSTRACT

Objective: Previously, we showed that the phosphatidylinositol-3 kinase (PI_3K) pathway mediates the anti-apoptotic effects of IGF-I in human neutrophils independently of its down-stream target Akt. In this study, we investigated whether IGF-I regulates Tec kinase, an alternative down-stream target of PI_3K, in neutrophils and whether this molecule is able to affect apoptosis.
Design: We investigated the translocation of Tec kinases in neutrophils after stimulation with IGF-I. Furthermore, we transiently and stably transfected Hek293T cells with constructs expressing different forms of Tec kinase and measured the level of cell survival and apoptosis/necrosis through trypan blue exclusion test and Annexin-V/propidium iodide labelling, respectively.
Results: We show that IGF-I stimulates the translocation of Tec kinase to the membrane in neutrophils in a PI_3K dependent matter. Overexpression of Tec kinase augments cell survival by inhibition of necrosis. The pro-survival effect is attenuated by the deletion of the kinase domain but not by inactivation of this domain by a single amino acid substitution.
Conclusion: Tec kinase can act as a prosurvival factor and is regulated by IGF-I in human neutrophils through PI_3K activation.

1. Introduction

Previously, we showed [1] through inhibitor studies that the phosphatidylinositol-3 kinase (PI_3K) pathway, but not the MEK-ERK pathway mediates the anti-apoptotic effects of IGF-I in human neutrophils, and that the signalling mechanism of IGF-I is different from the one used by GM-CSF, another PI_3K dependent anti-apoptotic agent. IGF-I did not operate through Akt in contrast to GM-CSF which stimulated this process in a PI_3K dependent-manner [2]. PI_3K has several downstream targets other than Akt which may be involved in inhibition by IGF-I of neutrophil apoptosis, including Tec family kinases.

In 1993, the importance of Tec family kinases came into light when several research groups discovered that a point mutation in the gene encoding one Tec family member, Btk (Bruton's tyrosine kinase), leads to immunodeficiency diseases in humans (X-linked agammaglobulinemia (XLA)) and mice (X-linked immunodeficiency (Xid)) [3]. Tec family kinases are not only expressed in hematopoietic cells but also outside of the hematopoietic lineage. For instance, Tec kinase was initially discovered in hepatocellular carcinoma, but was subsequently found to be expressed in all hematopoietic cells and in normal liver and kidney cells [4].

Tec kinases belong to a non-receptor tyrosine kinase family consisting of at least 5 members, and three of them are expressed by neutrophils (Tec, Btk and Bmx) [5]. Tec family kinases are abundantly expressed in the cytoplasm and predominantly unphosphorylated (inactivated) in unstimulated cells (resting state). Before acting as a tyrosine kinase protein, Tec kinase has to be translocated to the membrane, which involves the phosphorylation of PIP2 by PI_3K and subsequent recruitment of Tec kinase to PIP3 through its pleckstrin homology (PH) domain [6]. Subsequently, Tec kinase can be activated by phosphorylation of tyrosine residues by members of the Src-kinase family, including Lyn and Fyn kinase resulting in an active kinase.

In neutrophils, translocation of Tec kinase is induced by the chemoattractant fMLP, by the cell wall component of gram$^-$ bacteria lipopolysacharide (LPS) and by cross linking of the Fc receptor CD16b via activation of PI_3K [5,7,8]. Furthermore, Tec kinase can be activated in other cell types in response to ligation of antigens to the BCR (B-cell receptor) [9] and TCR (T-cell receptor) [10] and a wide range of other stimuli including growth factors (hepatocyt growth factor (HGF) [11], macrophage colony stimulation factor (M-CSF) [12], granulocyt colony stimulation factor (G-CSF) [13], granulocyt-macrophage colony stimulation factor (GM-CSF) [14], epidermal growth factor (EGF) [15], stem

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cell factor (SCF) [16]) and cytokines ((interleukin (IL) 3 [17], IL-6 [18], and IL-8 [5])).

Aside from functional roles of Tec kinase in regulation of cell proliferation, expression of cytokines, migration, phagocytosis and differentiation, Tec kinase and especially the other members of the Tec kinase family are involved in the regulation of cell survival. For instance, Melcher et al. [12] found that there was an impaired survival rate in $Tec^{-/-}Btk^{-/-}$ macrophages compared to the wild-type macrophages. Although the $Tec^{-/-}$ and $BTK^{-/-}$ macrophages showed a small reduction in number of cells, the combined knockout showed a 75% reduction in cell numbers. There is a further lack of direct evidence for the involvement of Tec kinase in the regulation of cell survival, but studies of other Tec kinase family members strongly suggest that they can regulate apoptosis. Mutations in the Btk gene are believed to lead to inappropriate apoptotic cell death of pre-B-cells leading to XLA [19,20]. Accordingly, in the murine variant of the disease (Xid), in contrast to wild-type cells, Xid B-cells underwent apoptotic cell death after stimulation with anti-IgM [21]. More recently, Honda et al. [22] showed that neutrophils from patients with XLA showed a significantly higher percentage of apoptotic cells after stimulation with TNFα and fMLP compared to control neutrophils. Furthermore, Petro et al. [23] provided evidence that in mice splenocytes, Btk targets NF-κB to activate the Bcl-x promoter through PLCγ2 and Vassilev et al. [24] described that in chicken B-lymphoma cells (DT40) Btk binds to Fas receptor were it prevents the Fas–Fadd interaction, which is essential in the DISC (death inducer signalling complex) for the recruitment of pro-caspase-8 and further downstream signalling. Our results and observations from the literature, prompted us to investigate the role of Tec kinases in cell survival and its regulation by IGF-I.

2. Materials and methods

2.1. Reagents

Recombinant human (rh) IGF-I was kindly provided by Lilly Research Laboratories (Indianapolis, IN). Contamination with endotoxin was not detectable using the Limulus Amebocyte Lysate assay (detection limit 1.25 pg/μg; Biowhittaker, Walkersville, MD). GM-CSF was obtained from PeproTech (London, UK). RMPI-1640 with glutamax-I, Dulbecco's modified Eagle medium (DMEM) with glutamax-I, penicillin/streptomycin (P/S) and fetal calf serum (FCS) were obtained from Invitrogen (Merelbeke, Belgium). Recombinant human transferrin, selenium, wortmannin, LY294002, dimethylsulfoxide (DMSO), blasticidin-S hydrochloride, propidium iodide (PI) and bovine serum albumin (BSA) were obtained from Sigma (Bornem, Belgium). [Methyl-^{3}H]-thymidine and horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG were obtained from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). FuGENE® 6 transfection reagent was obtained from Roche (Mannheim, Germany). Trypan blue was obtained from Merck Chemicals (Darmstadt, Germany) and the Annexin-V/FITC kit was purchased from Bender Medsystems (Vienna, Austria). Rabbit anti-Tec was from Upstate (Milton Keynes, UK). Goat anti-Btk, anti-Bmx, anti-actin and horseradish peroxidase (HRP) conjugated donkey anti-goat were obtained from Santa Cruz Biotechnology (Boechout, Belgium) while rabbit anti-Akt and rabbit anti-phospho-Akt (pS^{473}) were obtained from Biosource (Nivelles, Belgium).

2.2. Cell culture

Human neutrophils were purified from heparinized venous blood drawn from healthy donors as described previously [25]. Informed consent was obtained from all blood donors and the research protocol has been approved by the local ethical committee. Freshly isolated neutrophils were suspended in serum free medium (SFM: RPMI 1640 with glutamax-I, supplemented with 0.1% BSA (A-2153), 100 U/ml penicillin and 100 μg/ml streptomycin). BSA (A-2153) was of standard grade quality and selected by us, because it contained undetectable levels of IGF-I (<0.05 ng/mg). For the translocation experiments, neutrophils were treated in eppendorf tubes (Eppendorf AG, Hamburg Germany) in a pre-warmed (37 °C) water bath. Kinase inhibitors or vehicle (DMSO) were always applied 30 min before addition of IGF-I or GM-CSF.

Human embryonic kidney cells (Hek293T) used for all experiments were obtained from R. Beyaert (University of Ghent, Belgium). Hek293T cells were grown in DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin (P/S) supplemented with 10% FCS in a humidified 5% CO_2 atmosphere at 37 °C. To avoid interference with growth factors and IGF binding proteins from serum, we utilized SFM to perform our experiments.

2.3. Transient transfection

Full length Tec kinase (mouse Type IV) inserted at BamHI-EcoRI of pBluescript was cut out by XbaI and EcoRI and ligated to pSSRα vector at XbaI-EcoRI (pS-Tec) [17]. The empty PSSRα plasmid (pS-Vec) was used as a negative control. A Tec kinase deleted plasmid (BSR-TecKD) and a Tec kinase mutated plasmid (BSR-TecKM) were used to study the role of the kinase domain, and Tec kinase without the SH3 domain was used as a constitutively active Tec kinase (BSR-TecSH3). The latter plasmids carry a blasticidin-S (BSR) selection gene that allowed us to recover BSR-gene expressing Hek293T cells.

For transfection with pSSRα-Tec constructs, 0.5×10^6 Hek293T cells were seeded in a 24-well plate (Greiner, Wemmel, Belgium) in 1 ml culture medium (DMEM/10% FCS without antibiotics). The cells were grown overnight to allow adherence in humidified 5% CO_2 atmosphere at 37 °C. Then, the cells were transfected with 2 μg of pSSRα-Tec plasmid or other construct of interest using FuGENE® 6 reagent for 72 h. As a control, Hek293T cells were transfected with an empty pSSRα vector.

2.4. Stable transfection

Hek293T cells were transfected as described above with the plasmids containing the blasticidin-S Resistance (BSR) gene and harvested 72 h post-transfection (similar to transient transfection). The cells were diluted to a concentration of 1 cell/well in a 96 well plate. Next, the cells were allowed to attach for 24 h in normal culture medium before adding the selective antibiotic. Blasticidin-S (10 μg/ml) was used to positively select the cells containing the plasmid of interest. As a control, non-transfected Hek293T cells were also grown in the selective medium. The medium was changed every 2–3 days in the first week and every 5 days during the following 2 weeks. After 2 weeks, clones were selected and expanded in 250 ml culture flasks (Cellstar, Greiner). Transfected cells from the different clones were then harvested and levels of wild type and mutated Tec kinase proteins were assessed by western blotting. Control cells that were not transfected died within one week upon exposure to 10 μg/ml blasticidin-S.

2.5. Immunoprecipitation assay

Immunoprecipitation was performed on membrane fractions obtained from $40*10^6$ neutrophils. Cells were pre-incubated for 30 min in SFM (37 °C) and subsequently stimulated for 20 s with either vehicle or 300 ng/ml IGF-I. Stimulation was stopped by addition of 900 μl ice-cold KCl relaxation buffer and membrane fractions were isolated as previously described [1]. Subsequently, membranes were lysed in 100 μl buffer (50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1% NP40, 2.5 mM sodium orthovanadate, 10 mM $Na_4P_2O_7$, 50 mM NaF, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml soybean trypsin inhibitor, 50 mM pepstatin A and 1 mM AEBSF) for 10 min on ice. For immunoprecipitation 2 μg of anti-Tec kinase antibody