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p38 MAPK as a negative regulator of VEGF/VEGFR2 signaling pathway in serum deprived human SK-N-SH neuroblastoma cells

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Abstract

Evidence suggests that vascular endothelial growth factor (VEGF) mediates neuroprotection to prevent an apoptotic cell death. The p38 mitogen activated protein kinase (MAPK) pathway is implicated as an important mediator of neuronal apoptosis but its role in VEGF-mediated neuroprotection is unclear. Herein, we show that treatments with the p38 MAPK inhibitor, SB202190, enhanced VEGF-mediated survival in serum deprived SK-N-SH neuroblastoma cells by decreasing caspase-3/7 activation while increasing the phosphorylation of the extracellular signal-regulated kinase (ERK1/2) and Akt signaled through the VEGF receptor, VEGFR2. A blockade of VEGFR2 signaling with a selective inhibitor, SU1498 or gene silencing with VEGFR2 siRNA in SB202190 treated cells abrogated this prosurvival response and induced high activation levels of caspase-3/7. These findings suggested that the protection elicited by p38 MAPK inhibition in serum starved cells was dependent on a functional VEGF/VEGFR2 pathway. However, p38 MAPK inhibition attenuated caspase-3 cleavage in SU1498/SB202190 treated cells, indicating that p38 MAPK and caspase-3 only contributed in part to the total levels of caspase-3/7 induced by VEGFR2 inhibition. Pretreatments with the pan caspase inhibitor, z-VAD-fmk, prevented the apoptosis induced by VEGFR2 inhibition and promoted survival in serum starved cells irrespective of p38 MAPK inhibition. Collectively, our findings suggest that p38 MAPK exerts a negative effect on VEGF-mediated signaling through VEGFR2 in serum starved neuroblastoma cells. Furthermore, VEGF signals protection against a caspase-mediated cell death that is regulated by p38 MAPK-dependent and -independent mechanisms.

Introduction

Members of the mitogen activated protein (MAP) kinase family, ERK1/2 (extracellular signal-regulated kinase), SAPK1/JNK (stress activated protein kinase-1/c-Jun NH2-terminal kinase) and p38 MAPK (stress activated protein kinase-2), relay signals generated by growth factors, cytokines and stressful stimuli in neuronal cells [5,15]. Whereas growth factors stimulate the ERK1/2 cascade to regulate cellular processes such as proliferation, differentiation and survival, stressful stimuli stimulate the p38 MAPK pathway to induce cell death [15]. In neuronal cells, p38 MAPK pathway is linked to caspase activation and apoptosis induced by glutamate, β -amyloid, dopamine, 6-hydroxydopamine and cell death receptor activation [3,8, 11,12,30]. Moreover, a blockade of p38 MAPK signaling can also provide neuroprotection in vivo [2,4,28].

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Vascular endothelial growth factor (VEGF) is a well-established angiogenic and survival factor in endothelial cells that also possesses neuroprotective properties [27]. In endothelial cells, VEGF signaling through VEGFR2 promoted survival by preventing caspase-3 cleavage and p38 MAPK phosphorylation [25] while p38 MAPK inhibition enhanced ERK1/2 activation by VEGF [9]. In stressed neuronal cells, VEGF signals survival through its cognate receptor VEGFR2 and the downstream activation of the PI3K/Akt and MEK/ERK1/2 pathways [16, 27] and by suppressing the activation of caspase-3 [10,16] and p38 MAPK [13]. However, the exact role of p38 MAPK in VEGF-mediated signaling under stressful conditions is unclear since VEGF was shown to stimulate p38 MAPK after brain hypoxia [9] and inhibit its activation after retinal ganglion cell axotomy [13]. Using human SK-N-SH neuroblastoma cells as a neuronal model, we showed previously that serum deprivation induces an upregulation in the expression of VEGF and VEGFR2 which function concomitantly to signal survival through the activation of the PI3K/Akt and MEK/ERK1/2 pathways [7]. To extend these studies, we investigated whether p38 MAPK influenced the signaling of VEGF-mediated pathways that counteract caspase activation in serum deprived SK-N-SH cells. We show that p38 MAPK inhibition decreased caspase-3/7 activation in serum deprived SK-N-SH cells while enhancing the survival and phosphorylation of Akt and ERK1/2 mediated by VEGF through VEGFR2. Our findings also revealed that signaling through VEGFR2 protects against apoptotic pathways that are regulated in part by p38 MAPK.

Materials and methods

Materials

Recombinant human VEGF₁₆₅ was obtained from PeproTech Inc (Rocky Hill, NJ). SU1498, and SB202190 were obtained from EMD Biosciences Inc (San Diego, CA) and z-VAD-fmk were obtained from Biomol International (Plymouth Meeting, PA, USA).

Cell Culture

Human neuroblastoma SK-N-SH cells were maintained at 37°C in DMEM-F10 (1:1) supplemented with 5% fetal bovine serum (FBS) (Invitrogen Corporation, Carlsbad, CA, USA). For each experiment, cells were grown to 80% confluency followed by 48 hr of serum starvation and treated as indicated.

RNA Interference

Cells were transfected with 20 µM of KDR/Fik-1/VEGFR2 SMARTpool® siRNA duplexes (Dharmacon RNA Technologies, Lafayette, CO, USA) according to the manufacture's directions. Cells were then treated as indicated and incubated for 48 hr under serumfree conditions. A mixture of non-specific siRNA duplexes served as a negative control.

Cell Viability

Cells were plated in 96-well microtiter plates and treated as indicated under serum or serumfree conditions for 48 hr at 37°C. Cell viability was determined using a colorimetric MTS assay (Promega Corp, Madison, WI, USA) and quantified according to manufacturer's instruction. Survival measurements are expressed as the percent of the respective untreated control.

Reverse Transcriptase PCR (RT-PCR)

Total RNA was purified and reverse transcribed using gene specific primers for VEGF₁₆₅ and VEGFR2 as described previously [7].

Caspase-3/7 Activities

Cells were plated in a 96-well format and assayed for caspase activity using the fluorescence cell-based Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega) according to manufacturer's directions. Caspase activity was quantified using the Molecular Dynamics Typhoon™ 9410 Imaging System with ImageQuant software (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Data were normalized as fluorescent units/ μ g protein.

Nuclei Staining

Serum starved cells were treated as indicated for 48 hr at 37°C and then washed in PBS, fixed with 3.7% paraformaldehyde (20 minutes) at 37°C and stained with 1 μ g/ml of the chromatin-specific dye Hoechst 33342 (Sigma) for 20 minutes. Images of stained nuclei were captured at 60 $\times$ magnification using a Nikon OPTIPHOT-2 fluorescent microscope.

Protein Extraction and Western Blotting

Total cell lysates were prepared from serum starved cells as indicated using a lysis buffer [22] and Western blot analyses as described previously [7].

Statistical Analyses

Data are expressed as the mean $\pm$ SEM of experiments that were replicated at least three times. Statistical significance was assessed either by a one-way ANOVA followed by pairwise contrasts (Bonferroni analysis). A difference resulting in $P < 0.05$ is considered significant.

Results

In these studies, human SK-N-SH neuroblastoma cells were employed as a model to investigate whether p38 MAPK influenced VEGF-mediated survival in the neuronal response to serum deprivation [7]. SK-N-SH cells exhibit a neuronal phenotype and have been used extensively to assess potential neuroprotective mechanisms against numerous insults including serum deprivation [1]. To this end, cells were incubated for 48 hr with and without serum and treated with VEGF and a selective inhibitor of p38 MAPK, SB202190 either alone or in combination (Fig. 1A). Treatments with either SB202190 or exogenous VEGF increased viability only in serum starved cells and this effect was significantly enhanced when both were added in combination. Data are plotted relative to untreated serum or serumfree controls of 100%. Since p38 MAPK inhibition enhanced VEGF-mediated survival in serum starved cells, we examined the effects of a selective inhibitor of VEGFR2, SU1498, on the same experimental conditions shown in Fig. 1 (Fig. 1B). These experiments also included an assessment of SU1498 treatments on SB202190-induced effects in the absence of VEGF. VEGFR2 inhibition abrogated the survival induced by p38 MAPK inhibition only in serum deprived cells irrespective of the inclusion of VEGF. Similarly, the survival elicited by SB202190 was also diminished in serum deprived cells transfected with siRNA that specifically silence VEGFR2 gene expression (Fig. 1C). However, VEGF or p38 MAPK inhibition did not reduce p38 MAPK phosphorylation in serum deprived cells (Fig. 1D) nor did p38 MAPK inhibition affect the upregulation in VEGF or VEGFR2 induced by serum deprivation in SK-N-SH cells (Fig. 1E).

Since PI3K/Akt and MEK/ERK1/2 pathways are downstream effectors of VEGFR2-directed neuroprotection in serum starved SK-N-SH cells [7], we evaluated whether p38 MAPK inhibition affected their activation by VEGF. Western blot analyses revealed that treatments with SB202190 alone increased Akt and ERK1/2 phosphorylation to levels that were, respectively, 2 and 50 fold greater than serumfree controls (Fig. 2A, compare lanes 1 and 3) and 2-fold greater than VEGF treated cells (compare lanes 2 and 4) without changing their levels of total protein. Unlike Akt, exogenous VEGF increased ERK1/2 phosphorylation in

untreated cells by 2.5-fold (Fig. 2A, compare lanes 1 and 2). In SB202190 treated cells, exogenous VEGF did not mediate a further increase in Akt and ERK phosphorylation (Fig. 2A, compare lanes 3 and 4), suggesting that both kinases reach a threshold of VEGF-mediated activation with p38 MAPK inhibition alone. However, VEGFR2 inhibition abrogated Akt and ERK1/2 activation in VEGF treated cells irrespective of p38 MAPK inhibition (Fig. 2A, lanes 5 and 6). Similarly, the SB202190-mediated phosphorylation of ERK1/2 was attenuated in cells transfected with VEGFR2 siRNA (Fig. 2B, lane 4).

We then examined whether the increased viability in SB202190 or VEGF treated serum starved cells (Fig. 1A) was paralleled by changes in caspase-3/7 activation. Indeed, treatments with SB202190 or VEGF decreased caspase activation to levels that were significantly lower when both treatments were administered in combination (Fig. 3A). Conversely, the cell death induced by VEGFR2 inhibition (Fig. 1B) correlated with a significant increase in caspase-3/7 activity independent of p38 inhibition (Fig. 3B) and exogenous VEGF (data not shown). A further assessment of caspase activation revealed that SU1498 (Fig. 3B) or VEGFR2 siRNA (data not shown) induced the processing of the proform (35 kDa) of caspase-3 into 17 and 19 kDa fragments which was reduced by p38 MAPK inhibition (Fig. 3B, compare lanes 2 and 4). Measurements of reduced caspase-3 cleavage in SB202190 (or VEGF, data not shown) serum starved cells were inconclusive due to an inability to detect this processing in the untreated controls (Fig. 3B lane 1). Pretreatments with the pan caspase inhibitor z-VAD-fmk in serum starved cells treated with SU1498 alone or in combination with SB202190 increased viability to levels that resembled the untreated (100%) control (Fig. 4A) and abrogated the formation of nuclear chromatin condensation (Fig. 4B; compare *arrows* in top panel with bottom panel).

Discussion

In this report, we establish a functional link between VEGF-mediated survival and the regulation of caspase-3 activation by p38 MAPK in serum deprived human SK-N-SH neuroblastoma cells. We base our conclusion on the demonstrations that p38 inhibition prevented caspase-3 cleavage induced by VEGFR2 inhibition (Fig. 3B) and enhanced the cell viability (Fig. 1A), the activation of ERK1/2 and Akt (Fig. 2A) and the decrease in caspase-3/7 activity (Fig. 3A) mediated by VEGF without affecting the increase in VEGF and VEGFR2 gene expression induced by serum deprivation (Fig. 1E). The fact that SB202190 promoted survival without reducing p38 MAPK phosphorylation (Fig. 1D) was previously observed in degenerating motor neurons [4] and in endothelial cells [9]. This observation is also consistent with the mechanism of action of SB202190, which inhibits p38 MAPK activity but not kinase phosphorylation [26]. Accordingly, these results implicate p38 MAPK as a negative regulator of VEGF-mediated neuroprotection and are consistent with previous reports with endothelial cells that activated p38 MAPK exerts opposing effects on FGF-2 or VEGF-mediated survival [9,16].

The demonstration that VEGFR2 inhibition abolished SB202190-mediated survival (Figs. 1B and 1C), ERK1/2 and Akt phosphorylation (Fig. 2) and caspase activation (Fig. 3B and data not shown) also suggests that the protection induced by p38 MAPK inhibition requires a functional VEGF/VEGFR2 pathway. The finding that SB202190 attenuates caspase-3 cleavage in SU1498 treated serum deprived cells also implicates p38 MAPK as a regulator of the apoptosis suppressed by VEGF signaling. However, the fact that caspase-3/7 activation (Fig. 3B), chromatin condensation (Fig. 4B) and cell death (Fig. 4A) persist when caspase-3 cleavage is blocked suggests that VEGFR2 inhibition induces distinct cell death mechanisms in serum deprived cells whereby p38 MAPK mediates caspase-3 activation and a p38 MAPK-independent pathway that activates disproportionately high levels of caspase-7. This discrepancy in the levels of caspase activity may reflect why caspase-3/7 activation but not caspase-3 cleavage is observed in untreated and SB202190 serum starved treated cells. It is

conceivable that p38 MAPK regulates caspase-3 activation through caspase-8 directly [3], death receptor activation [8] or the translocation of the proapoptotic protein Bax to the mitochondria [6]. Alternatively, the p38 MAPK-independent mechanism may involve a direct activation of caspase-7 by caspase-9 [19], calpain [23], granzyme B or cathepsin G [29] to function independently of caspase-3. For example, caspase-7 is required to mediate endoplasmic stress which can be induced by serum deprivation [21]. The demonstration that VEGF reduces caspase-3/7 activation (Fig. 3A) but not p38 MAPK phosphorylation (Fig. 1D) suggests that VEGF signals its antiapoptotic properties irrespective of p38 MAPK activation. In this context, VEGF may relay protection through an Akt-mediated upregulation in the antiapoptotic protein Bcl-2 [14] or through an ERK1/2 or Akt-mediated inactivation of proapoptotic proteins by phosphorylation [5]. Consequently, the VEGF/VEGFR2 pathway may signal a global neuroprotection against neuronal apoptosis but further analyses are required to delineate these mechanisms.

Evidence suggests that PI3K/Akt and MEK/ERK1/2 are critical downstream effectors of VEGF-mediated neuroprotection [16,25,27]. The fact that the survival induced by p38 inhibition is coincident with increased activation levels of ERK1/2 and Akt through VEGFR2 (Fig. 2) suggests that p38 MAPK modulates VEGF-mediated signaling pathways that counteract cell death in serum starved SK-N-SH cells. These findings are supported by studies in serum starved endothelial cells showing that p38 MAPK inhibition reduces apoptosis and enhances VEGF-dependent ERK1/2 activation to mediate angiogenesis [9] or survival [25]. The 50-fold increase in ERK1/2 activation by p38 MAPK inhibition (Fig. 2A) suggests that ERK1/2 alone is highly sensitive to perturbations by p38 MAPK activity. Indeed, ERK1/2 inhibition alone can sensitize neuronal cells toward an apoptotic cell death [18] while ERK1 overexpression is sufficient to rescue SK-N-SH cells from the caspase-dependent cell death induced by VEGFR2 inhibition [7]. The fact that VEGFR2 inhibition induces a loss in ERK1/2 phosphorylation (Fig. 2A) that correlates with increased caspase activity (Fig. 3B) and cell death (Fig. 1B) is also consistent with reports showing that a downregulation in ERK1/2 activation accompanies p38 MAPK-mediated apoptosis in both neuronal and nonneuronal cells [17,20,24].

In summary, our findings provide new insight on the role of p38 MAPK in VEGF-mediated survival when neuronal cells are exposed to apoptotic stimuli. These findings suggest that the VEGF/VEGFR2 pathway signals neuroprotection to prevent a caspase-mediated cell death regulated by p38 MAPK-dependent and -independent mechanisms.

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Fig. 1.

VEGF and p38 MAPK have opposing effects on the survival of serum deprived neuronal cells. Cell viability was measured following 48 hr incubations in the presence and absence of serum with A, 10 μ g/ml VEGF (VE) and 10 μ M SB202190 (SB) alone or in combination (SB/VE), or VE with B, 10 μ M SU1498 (SU) and SB alone and in combination (SB/SU) or C, 20 μ M of nonspecific siRNA duplexes (control; siC) and 20 μ M of VEGFR2 silencing duplexes (siRNA) alone and in combination with SB (siRNA/SB). D, Total lysates from serum starved cells treated without or with VE or SB were analyzed by Western blotting for the phosphorylated and total forms of p38 MAPK. E, Total RNA from serum treated and serum starved cells incubated without and with SB were analyzed by RT-PCR for VEGF₁₆₅ or VEGFR2 expression levels. Results represent the $\pm$ S.E.M. of the percent cell viability relative to untreated control cells (100%), respectively, from at least three independent experiments. Significance is (***) $P < 0.001$ for cell viability between SB/VE versus VE or SB in A and SB versus SU or SB/SU in B, (**) $P < 0.01$ between siC versus siRNA and (*) $P < 0.05$ between siC/SB versus siRNA/SB in C.

Fig. 2.
p38 MAPK inhibition augments VEGF-mediated activation of ERK1/2 and Akt. SK-N-SH cells were serum starved and treated as indicated with the concentrations given in the legend to Fig. 1. Total lysates were analyzed by Western Blotting for the phosphorylated and total forms of Akt (A), ERK1/2 (A and B) with actin in A or total ERK1/2 in B as the protein loading controls.

Fig. 3. VEGF and p38 MAPK inhibition regulate caspase activation. Serum starved cells were treated as indicated with the concentrations given in the legend to Fig. 1. **A**, Cells were then analyzed for caspase-3/7 activity or **B**, caspase-3/7 activity together with caspase-3 cleavage by Western blotting. Results represent the $\pm$ S.E.M. of the caspase activity (units/ μ g protein) relative to untreated control cells (100%) from at least three independent experiments. Significance is (***) $P < 0.001$ for caspase activities between SF versus VE, SB, or SB/VE; (* $P < 0.05$) between SB/VE versus VE or SB in **A**. (***) $P < 0.001$ between SB versus SU or SB/SU in **B**.

Fig. 4. VEGFR2 inhibition induces a caspase-dependent cell death. Serum starved cells were pretreated without and with 20 μ M of the caspase inhibitor z-VAD-fmk (z-VAD) and then treated as indicated with the concentrations given in the legend to Fig. 1. **A**, Cells were then analyzed for cell viability or **B**, by Hoechst 33342 staining for nuclear chromatin condensation (arrows). Scale bar is 20 μ m. Results represent the $\pm$ S.E.M. of the percent cell viability relative to untreated control cells (100%) from at least three independent experiments. Significance is (***) $P < 0.001$ for cell viability between z-VAD/SB versus SB/SU or z-VAD/SB/SU, SB/SU versus z-VAD/SB/SU and SU versus z-VAD/SU in **A**.