Control of late apoptotic events by the p38 stress kinase in L-glutamine-deprived mouse hybridoma cells

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L-Glutamine (Gln) starvation rapidly triggers apoptosis in Sp2/0-Ag14 (Sp2/0) murine hybridoma cells. Here, we report on the role played by the stress-activated kinase p38 mitogen-activated protein kinase (MAPK) in this process. p38 activation was detected 2 h after Gln withdrawal and, although treatment with the p38 inhibitor SB203580 did not prevent caspase activation in Gln-starved cells, it reduced the occurrence of both nuclear condensation/fragmentation and apoptotic body formation. Similarly, transfection of Sp2/0 cells with a dominant negative p38 MAPK reduced the incidence of nuclear pyknosis and apoptotic body formation following 2 h of Gln starvation. Gln withdrawal-induced apoptosis was blocked by the overexpression of the anti-apoptotic protein Bcl-xL or by the caspase inhibitor Z-VAD-fmk. Interestingly, Bcl-xL expression inhibited p38 activation, but Z-VAD-fmk treatment did not, indicating that activation of this MAPK occurs downstream of mitochondrial dysfunction and is independent of caspases. Moreover, the anti-oxidant N-acetyl-L-cysteine prevented p38 phosphorylation, showing that p38 activation is triggered by an oxidative stress. Altogether, our findings indicate that p38 MAPK does not contribute to the induction of apoptosis in Gln-starved Sp2/0 cells. Rather, Gln withdrawal leads to mitochondrial dysfunction, causing an oxidative stress and p38 activation, the latter contributing to the formation of late morphological features of apoptotic Sp2/0 cells. Copyright © 2012 John Wiley & Sons, Ltd.

KEY WORDS—p38 stress kinase; apoptosis; glutamine; mitochondrial dysfunction; oxidative stress; nuclear condensation; apoptotic bodies

INTRODUCTION

In the 1950s, Harry Eagle recognized L-glutamine (Gln) as the single most important factor required in culture media to maintain and promote cell proliferation.¹ Although Gln is generally considered as a non-essential amino acid, the observation that it can be limiting under severe stresses²–⁴ has led to its re-classification as a conditionally essential amino acid. In addition to its role as an energy source, Gln is required for the biosynthesis of glutathione (GSH), polyamines, amino sugars, NAD(P) co-factors and nucleotides.⁵–⁷ Furthermore, many signal transduction pathways have been shown to be modulated by Gln, including the mammalian target of rapamycin, the extracellular signal-regulated kinase and the c-Jun N-terminal kinase pathways.⁸ With this broad spectrum of targets influenced by Gln, it is not surprising that this amino acid has been reported to influence numerous cellular processes, including survival.⁹–¹¹ Gln supplementation has been shown to reduce the incidence of apoptosis when triggered by heat shock,¹² irradiation¹³ or c-Myc overexpression.¹⁴ In addition, Gln withdrawal induces apoptosis in RIE-1, Sp2/0-Ag14, CEM and HL-60 cells.⁹,¹⁰,¹⁵,¹⁶ The anti-apoptotic nature of Gln was demonstrated to be unrelated to its ability to be used as an energy source for the cell,¹⁷,¹⁸ suggesting that Gln modulates cell survival at least in part by acting upon signalling pathways.

We have demonstrated that the Sp2/0-Ag14 hybridoma (Sp2/0) is strictly dependent on Gln for its survival, making it a useful model for studying the molecular and cellular events responsible for the anti-apoptotic function of Gln.¹⁰ Sp2/0 cells commit to undergo apoptosis within 2 h of Gln deprivation, a phenomenon that involves mitochondrial dysfunction and the activation of caspase-3 and caspase-9, all within 1 h of Gln withdrawal.¹⁰ Importantly, this phenomenon is only observed upon Gln starvation, as depriving Sp2/0 cells of any of the other 19 amino acids does not trigger rapid apoptosis.¹⁰ The biochemical basis for the acute sensitivity of Sp2/0 cells to Gln withdrawal is currently unknown but may result from the combination of the low expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL, as well as a constitutively expressed c-Myc.¹⁹,²⁰ However, the molecular and cellular events responsible for the rapid death of Sp2/0 cells upon Gln starvation are still obscure.

The p38 mitogen-activated protein kinase (p38 MAPK) is activated by numerous environmental stresses including osmotic stress, hypoxia, heat shock, UV radiation and oxidative stress.²¹,²² p38 MAPK is part of an important kinase cascade that is initiated by the stress-specific activation of a number of MAPK kinase kinases (MAP3Ks), such as apoptosis...
signal-regulating kinase-1 (ASK1) and transforming growth factor β-activating kinase-1 which, in turn, catalyze the phosphorylation-mediated activation of the p38-specific MAPK kinases (MAP2Ks) MKK3 and MKK6.23 The latter activate p38 MAPK through dual phosphorylation on residues Thr 180 and Tyr 182. Activated p38 MAPK has been shown to modulate many cellular processes including cell differentiation, growth and death.24–26 The induction of apoptosis by p38 occurs through a number of ways, including the modulation of gene expression,27,28 the regulation of the activity of proteins involved in triggering cell death29–31 or in controlling late apoptotic events such as membrane blebbing.32,33

Interestingly, our group has previously shown that Gln deprivation caused an oxidative stress in Sp2/0 cells. Using the anti-oxidant N-acetyl-L-cysteine (NAC), we demonstrated that, although an oxidative stress was not required for the induction of cell death, the formation of apoptotic bodies was significantly decreased.34 Because the p38 MAPK is known to modulate the cellular response to oxidative stress,35,36 we investigated the possibility that this signalling pathway plays a role in the apoptotic process in Gln-starved Sp2/0 cells. In this report, we show that the p38 MAPK pathway does not participate in the initiation of the death programme but rather contributed, in an oxidative-stress-dependent manner, to the formation of membrane blebbing and nuclear condensation.

MATERIALS AND METHODS

Reagents

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich Canada (Oakville, ON). Gln (200 mM) and NAC (1.5 M) stock solutions were made fresh in phosphate-buffered saline (PBS; 9.1 mM Na2HPO4, 1.7 mM NaH2PO4, 150 mM NaCl, pH 7.4) and adjusted to pH 7.4. The pan-caspase inhibitor Z-VAD-fmk and the p38 MAPK inhibitor SB203580 (EMD Chemicals, San-Diego, CA) were dissolved in dimethyl sulfoxide (DMSO) as 10 and 5 mM stocks, respectively. The flag-tagged p38 MAPK dominant negative plasmid (p38agf)37 was a kind gift from Dr Jiahuai Han (Xiamen University, China).

Cell culture

Sp2/0-Ag14 murine hybridoma cells (CRL 1581) were obtained from the American Type Culture Collection (Rockville, MD). All cells were maintained in Iscove’s-modified Dulbecco’s medium (IMDM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5% FetalClone I (HyClone, Logan, UT) and 4 mM Gln (herein referred to as complete IMDM). Sp2/0 cells stably expressing Bcl-xL38 or p38agf were maintained in complete IMDM supplemented with 400 μg/ml G-418. Cell culture was conducted at 37 °C in a humidified atmosphere of 5% CO2.

When SB203580 was used, a 1 h pre-incubation of the cells with the drug was performed in complete IMDM. For the Gln deprivation experiments, exponentially growing cells were washed twice with PBS and resuspended in complete IMDM lacking Gln. At this point, SB203580, Z-VAD-fmk and/or NAC were added to 5, 10 and 15 mM respectively, and the cells were cultured for the specified time intervals. DMSO was added to the SB203580 and Z-VAD-fmk control groups, whereas PBS was used for the NAC controls.

To determine the viability of the Gln-starved Sp2/0 cells, samples were taken at the indicated time intervals. Gln was added to a final concentration of 4 mM, and the cells were cultured overnight. The number of viable and dead cells was then determined using the trypan blue dye exclusion assay.

Western blot analysis

Cytosolic and whole-cell protein extracts were prepared as described previously.10 The protein content was determined using the DC protein assay from Bio-Rad (Mississauga, ON), and the extracts were stored at −80 °C until needed. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon-P membrane (EMD Millipore Corporation, Billerica, MA). Transfer efficiency was verified by staining the membrane with Ponceau S. Western blot analysis was performed using the following primary antibodies (unless stated, antibodies were from Cell Signaling Technology, Danvers MA): rabbit anti-phospho-p38 MAPK (Thr180/Tyr182), rabbit anti-p38 MAPK, rabbit anti-phospho-MKK3/6 (Ser189/207), rabbit anti-MKK3/6, rabbit anti-caspase-9, rabbit anti-cleaved caspase-3, rabbit anti-DYKDDDK Tag, rabbit anti-cytochrome c, rabbit anti-HSP60 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rabbit anti-Bcl-xL (Santa Cruz Biotechnology Inc.). A goat anti-rabbit IgG secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology Inc.) was used as secondary antibody. Detection was performed by chemiluminescence using the Immobilon Western reagent (EMD Millipore Corporation) and the Fluorchem 8000 Imaging System (Alpha Innotech, San Leandro, CA).

Apoptosis assays

Nuclear condensation. Hoechst 33342 was added to Sp2/0 cells to a final concentration of 2 μg/ml, 30 min prior to the end of the experiment. The cells were then washed twice with PBS, resuspended in PBS and observed under fluorescence microscopy using a Leitz Diaplan microscope (Leica Microsystems, Richmond Hill, ON, Canada).

Membrane blebbing. Cells were taken at the indicated time intervals, placed on ice for 10 min and washed once in cold PBS. The cell pellets were then resuspended in PBS containing 6 μg/ml each of acridine orange and ethidium bromide, incubated at room temperature for 1 min and visualized by fluorescence microscopy.

Caspase activity assay. Caspase-3 activity was determined as described using a colorimetric enzyme assay (Biovision Inc, Mountain View, CA) following instructions from the manufacturer.
p38 MAPK activity assay

p38 MAPK activity was determined using the p38 MAP Kinase Assay Kit (Cell Signaling Technologies) following the instructions provided by the manufacturer.

Determination of cellular GSH levels

The cellular levels of GSH were determined as previously described using the ApoGSH GSH detection kit (Biovision Inc., Mountain View, Ca, USA), following the instructions provided by the manufacturer.

Statistical analysis

Statistical significance was determined using a one-way analysis of variance and Scheffe’s post hoc test.

RESULTS

p38 MAPK is activated in Gln-starved Sp2/0 cells

To determine whether the p38 MAPK pathway is activated in Gln-deprived Sp2/0 cells, we analyzed the phosphorylation status of p38 and its upstream activators MKK3/6 at different time intervals after Gln withdrawal. As depicted in Figure 1, the activating phosphorylation of MKK3/6 and p38 MAPK was detected when Sp2/0 cells had been starved of Gln for at least 1 h and was maximal at 2 h. Interestingly, the phosphorylation of MKK3/6 and p38 MAPK coincided with the loss of cell viability, suggesting that the p38 MAPK

![Figure 1](image1.png)

Figure 1. The p38 mitogen-activated protein kinase (MAPK) pathway is activated in L-glutamine (Gln)-deprived Sp2/0 cells. Sp2/0 cells were incubated in Gln-free complete Iscove’s modified Dulbecco’s medium (IMDM) for the indicated time intervals and processed for Western blot analysis of phosphorylated MKK3/6 and p38 MAPK. Pan-p38 MAPK and pan-MKK3/6 antibodies (which detect both the phosphorylated and non-phosphorylated forms) were also used to ensure that the observed changes in the phosphorylated forms of the proteins were not the result of global variation in protein level. Cells cultured for 120 min in complete IMDM were included as controls (lane C). As a measure of cell viability, cell samples were taken at the indicated time intervals, supplemented with Gln (4 mM final concentration) and cultured for 24 h. The percentage of cells with intact membranes was then determined using the trypan blue exclusion assay.

![Figure 2](image2.png)

Figure 2. SB203580 does not block caspase activation or the cytosolic release of mitochondrial apoptogenic proteins. SP2/0 cells were pre-treated for 1 h with SB203580 or dimethyl sulfoxide (DMSO), washed twice with phosphate-buffered saline and incubated for 2 h in complete Iscove’s modified Dulbecco’s medium supplemented or not with 4 mM L-glutamine (Gln). (A) Following the 2 h Glu starvation, Gln was added to the amino acid-deprived cells and incubated for 24 h. Cell viability was then measured using the trypan blue assay. (B) Whole-cell extracts were assayed for the presence of caspase-9, as well as cleaved caspase-3. Cytosolic extracts were prepared and probed for the presence of cytochrome c. (C) Caspase-3 activity. Results are the average ± standard deviation of three independent experiments.
pathway could play a role in the death programme triggered in Sp2/0 cells following Gln starvation.

**p38 MAPK modulates late apoptotic events**

To determine whether p38 MAPK activation is required for the initiation in Gln-starved Sp2/0 cells, we tested the effect of the potent p38 inhibitor SB203580 on cell viability, caspase activation as well as on the cytosolic release of the mitochondrial protein cytochrome c. Treatment of Sp2/0 cells with SB203580 did not change the sensitivity of the cells to 2 h of Gln starvation (Figure 2(A)). In agreement with our previous observations, Gln deprivation triggered the cleavage of both caspase-9 and caspase-3 into their active form, as well as the release of cytochrome c into the cytoplasm (Figure 2(B)). The addition of SB203580 had no effect on either caspase activation or the cytosolic release of cytochrome c (Figure 2(B)). Supporting these results, a caspase-3 activity assay showed no difference between SB203580 and DMSO treatment following 2 h Gln starvation (Figure 2(C)). These results indicate the p38 MAPK activity is not required for the initiation of the apoptotic death machinery in Gln-starved Sp2/0 cells.

Previous reports have shown that stress-induced p38 MAPK activation participates in the activation of late apoptotic events including nuclear condensation and membrane blebbing. We therefore tested whether p38 MAPK inhibition would affect the appearance of these morphological apoptotic features in Gln-deprived Sp2/0 cells. Cells treated with vehicle alone or SB203580 were cultured in the presence or absence of Gln for 2 h, and cells with apoptotic bodies or condensed nuclei were enumerated. Whereas the control groups cultured in the presence of Gln showed only a few cells with apoptotic bodies or nuclear condensation, Gln starvation led to a significant increase in both apoptotic body formation (Figure 3(A)) and nuclear pyknosis (Figure 3(B)). Interestingly, treating Sp2/0 cells with SB203580 significantly reduced the percentage of cells exhibiting apoptotic bodies or condensed nuclei. To confirm the data obtained with SB203580, we constitutively expressed a dominant negative p38 mutant (p38agf) in Sp2/0 cells (Figure 4(A)). The expression of the dominant negative p38 mutant did not alter the cellular sensitivity to Gln starvation (Figure 4(B)). Importantly, the expression of p38agf significantly reduced the number of Sp2/0 cells exhibiting apoptotic body and condensed nuclei following Gln starvation (Figure 4(C) and (D)). Thus, these data indicate that p38 MAPK is not required for the induction of the death machinery in Gln-starved Sp2/0 cells but is important for promoting late apoptotic events.

**p38 MAPK is activated downstream of mitochondrial dysfunction in a caspase-independent manner**

We next sought to further characterize the molecular events triggered by Gln starvation and which lead to the activation of p38 MAPK. Sp2/0 cells express low levels of Bcl-xL, a pro-survival protein that prevents apoptosis by preserving the integrity of mitochondria. Sp2/0 cells ectopically expressing Bcl-xL (Figure 5(A)) were protected from Gln-starvation-induced apoptosis, in contrast to the vectortransfected control that rapidly underwent cell death (Figure 5(B)). Interestingly, ectopic Bcl-xL expression was sufficient to prevent the phosphorylation and activation of p38 following Gln deprivation (Figure 5(C)), suggesting that the induction of the p38 MAPK pathway occurred downstream of mitochondrial dysfunction.

To determine whether p38 MAPK activation in Gln-deprived Sp2/0 cells required caspase activation, we used the pan-caspase inhibitor Z-VAD-fmk. Treating Sp2/0 cells with Z-VAD-fmk was sufficient to protect them against Gln-starvation-induced cell death (Figure 6(A)). However, Z-VAD-fmk did not prevent the activation of p38 MAPK...
following Gln deprivation (Figure 6(B)). All together, these data indicate that p38 MAPK activation in Gln-starved Sp2/0 cells occurs downstream of mitochondria in a manner that does not require caspase activation.

p38 MAPK is activated by an oxidative stress

In addition to caspase activation, mitochondrial dysfunction results in an increased production of reactive oxygen species (ROS), leading to oxidative stress. 40-43 Because Sp2/0 cells undergo an oxidative stress upon Gln starvation, 34 we used the ROS scavenger NAC to determine whether p38 MAPK activation is caused by an increased oxidative burden. In agreement with our previous report, 34 treating Sp2/0 cells with NAC led to a significant reduction of oxidative stress, as measured by the cellular levels of the reduced form of GSH (Figure 7(A)). Furthermore, and in agreement with findings from our laboratory, 34 NAC did not protect Sp2/0

cells from Gln-starvation-induced cell death (Figure 7(B)). However, NAC significantly reduced the phosphorylation of p38 MAPK upon Gln starvation (Figure 7(C)), indicating that the activation of this MAPK is caused by oxidative stress in Gln-deprived Sp2/0 cells.

**DISCUSSION**

Few studies have addressed the effect of Gln on the p38 MAPK pathway. Whereas two reports have shown an increase of p38 activation with increased Gln levels,44,45 two others demonstrated an inhibitory effect of Gln on the p38 MAPK pathway.46,47 Lagranha et al. showed that the increased apoptosis seen in rat neutrophils after physical exercise could be inhibited by Gln administration and that this effect was associated with a decrease in p38 MAPK activity.46 Sakiyama et al. also demonstrated an inhibitory effect of Gln on p38 activation, which was associated with a reduction in caspase-3 activation.47 However, these studies did not investigate the molecular mechanisms underlying the modulation of p38 MAPK by Gln. In this report, we used the Sp2/0 hybridoma, a cell line that is acutely dependent on Gln for its survival,10 to investigate the role which the stress-activated protein kinase p38 MAPK plays in the death programme triggered by Gln starvation.
Our results revealed that, although p38 MAPK is indeed phosphorylated and activated in Sp2/0 cells upon Gln starvation, blocking its function with SB203580 did not prevent cell death, caspase activation or the cytosolic release of the mitochondrial protein cytochrome c (Figure 2). Furthermore, we demonstrated, through the use of the p38 MAPK inhibitor SB203580 and the expression of a dominant negative p38 MAPK mutant protein, that p38 contributes to apoptotic body formation and nuclear condensation in Gln-deprived Sp2/0 cells (Figures 3 and 4). Our data also revealed that p38 MAPK activation was not blocked by a caspase inhibitor but that it could be prevented by Bcl-xL overexpression and by the antioxidant NAC, indicating that p38 MAPK activation occurred as a consequence of mitochondrial dysfunction and in a caspase-independent/ROS-dependant manner (Figures 5, 6 and 7). Therefore, we conclude that p38 MAPK is dispensable for the initiation of apoptosis in Gln-starved Sp2/0 cells, that it is activated downstream of mitochondrial dysfunction and that it contributes to morphological events occurring late in the death programme.

While being at odds with several reports implicating p38 MAPK in events leading to the outer mitochondrial membrane permeabilization (OMMP) and caspase activation, our data are in line with a number of other studies where this MAPK was shown to act downstream of mitochondria to modulate apoptotic membrane blebbing and nuclear condensation. Although the molecular events through which p38 MAPK regulates nuclear pyknosis remain to be clearly defined, the control of membrane blebbing involves the modulation of microfilament dynamics through the p38 MAPK-mediated phosphorylation of HSP27, a protein involved in the control of actin polymerization. In some of the studies reporting on a post-mitochondrial role of p38 MAPK in modulating apoptosis, cell death was triggered by c-Myc overexpression or upon nutrient/hypoxic stress. Interestingly, Sp2/0 cells exhibit deregulated c-Myc expression, and several genes modulated by Gln starvation in this and other cell lines also show altered expression following hypoxia or glucose starvation (e.g. gadd153).

The exact molecular mechanism underlying the activation of p38 MAPK in Gln-starved Sp2/0 cells remains to be uncovered. We show in this report that oxidative stress plays an important role in p38 activation (Figure 7). That p38 MAPK activation can be inhibited by NAC provides a direct explanation for our observation that NAC, while being ineffective in preventing cell death, significantly decreased apoptotic body formation in Gln-deprived Sp2/0 cells. Even though the exact cause of oxidative stress in Gln-deprived Sp2/0 cells remains to be firmly established, two mutually non-exclusive possibilities are likely at play. Firstly, through its conversion to glutamate, Gln is a precursor for the synthesis of GSH. Gln withdrawal is therefore expected to lead to a reduction in the intracellular levels of GSH, weakening the cell’s antioxidant defences and exacerbating the effects of increased ROS production. Such a decrease in intracellular GSH is indeed observed in Sp2/0 cells upon Gln starvation (Figure 7(A)). Secondly, we have shown that Gln starvation in Sp2/0 cells leads to the rapid recruitment of pro-apoptotic proteins such as Bax to the mitochondria and the release of the apoptogenic proteins cytochrome c and SMAC-Diablo. The latter is indicative of OMMP. OMMp is often followed by the dissipation of the mitochondrial transmembrane

Figure 7. p38 mitogen-activated protein kinase (MAPK) activation required an oxidative stress. (A) Sp2/0 cells were cultured for 3 h in complete Iscove’s modified Dulbecco’s medium supplemented or not with 4 mM L-glutamine (Gln) and/or 15 mM N-acetyl-L-cysteine (NAC). Glutathione (GSH) levels were then determined. Results are represented as the fold difference in GSH levels between Gln-starved cells versus the corresponding Gln-supplemented sample. (B) Sp2/0 cells were cultured for 3 h under the indicated conditions. Gln (4 mM) was then added to the Gln-deprived groups, cell culture was resumed for 24 h and the percentage of membrane-intact cells was determined using the trypan blue exclusion assay. (C) p38 MAPK activation as evidenced by Western blot analysis. Results are the average ± standard deviation of three independent experiments.
potential, leading to increased generation of ROS from the mitochondrial electron transport chain. In addition, ROS production can be increased through the caspase-mediated cleavage of components of the electron transport chain. Following the increased production of ROS, p38 MAPK is activated through the redox-dependent, thioredoxin-modulated activation of its upstream MAPK ASK1.

Interestingly, ASK1 has previously been shown to be modulated by Gln but through a different mechanism. Work with HeLa cells has demonstrated that Gln supplementation leads to ASK1 inhibition via its association with glutaminyl-tRNA synthetase bound to a Gln-charged tRNA. Notably, Gln starvation caused an accumulation of free tRNA, leading to the disruption of the ASK1-glutaminyl-tRNA synthetase interaction, ASK1 activation and increased sensitivity to Fas ligand-induced apoptosis. However, the authors failed to see an increase in p38 MAPK activation, either upon Gln starvation or Fas ligand treatment. Although the involvement of ASK1 in the activation of p38 MAPK in Gln-starved Sp2/0 cells remains to be determined, both the study by Ko et al. and the findings reported here indicate that Gln can modulate an important cell signalling pathway through a number of independent routes. Considering that GSH depletion and the ensuing oxidative stress have been observed in several situations of Gln starvation or limitation and that p38 MAPK signalling impinges on the regulation of cell survival, differentiation and proliferation, the p38 MAPK pathway is likely to be a major component of the signalling events involved in the cellular response to limiting Gln levels.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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