ASK1 resistant neuroblastoma is deficient in activation of p38 kinase

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Abstract

Apoptosis Signal-regulating Kinase 1 (ASK1) is known to either induce apoptosis or differentiation in various cell lines of neuronal origin. We analyzed the effect of the constitutively active mutant of ASK1 (ASK1-ΔN) in an adenoviral vector in four neuroblastoma cell lines, two murine, C1300 and NXS2, and two human, SH-SY5Y and IMR-32. Already after 24 h upon infection, C1300 and SH-SY5Y cells arrested in growth when judged by [³H]thymidine incorporation, and the majority of the cells demonstrated apoptotic appearance, which was confirmed by DNA-laddering in gel electrophoresis. In contrast, NXS2 and IMR-32 cell lines remained unaffected. Immunoblotting revealed strongly phosphorylated p38 MAPK accompanied by weakly phosphorylated JNK in C1300 and SH-SY5Y, whereas none of these kinases were activated by adenoviruses expressing the kinase negative ASK1 mutant or β-galactosidase. There was no expression of phosphorylated kinases in IMR-32 cells, but NXS2 showed a faint band of phosphorylated p38 MAPK. Addition of the p38 MAPK specific inhibitor, SB203580, protected C1300 and SH-SY5Y cells from apoptosis induced by ASK1-ΔN. The anti-neoplastic agent, paclitaxel, activates ASK1 and JNK, and promotes the in vitro assembly of stable microtubules. Addition of 10 nM paclitaxel sensitised the NXS2 cell line to ASK1-induced cell death. Our results indicate that ASK1 induces apoptosis in neuroblastoma cells mainly via the p38 MAPK pathway, and resistant neuroblastoma cells can be sensitised to ASK1 by paclitaxel. Cell Death and Differentiation (2001) 8, 1029–1037.

Keywords: neuroblastoma; ASK1; apoptosis; p38 MAPK; JNK

Abbreviations: ASK1, apoptosis signal-regulating kinase; ΔN, N-terminal deletion; ECL, enhanced chemoluminescence; HA, hemagglutinin; HRP, horseradish peroxidase; IL-1, interleukin-1; JNK, c-jun N-terminal kinase; NGF, nerve growth factor; p38 MAPK, p38 mitogen activated protein kinase; TNF, tumour necrosis factor; WT, wild-type

Introduction

Neuroblastoma occurs in the sympathetic nervous system, being one of the most common solid tumours of infancy.[1] The tumour progression is very heterogeneous, ranging from the most favourable case where the tumour differentiates to benign ganglioneuroma or spontaneously disappears[2], to the other extreme where the patient has disseminated disease at the time of diagnosis. Despite aggressive treatment involving surgical removal of primary tumour, multiagent regimes of chemotherapy and high-dose chemo- and radiotherapy followed by autologous bone marrow transplantation, the prognosis for such a patient is poor, with 5 years survival rate of less than 40%.[3] There is a need to develop new therapeutic approaches that are more effective and less toxic than the current regimes, improving the survival rate of patients with advanced neuroblastoma.

Programmed cell death occurs in the nervous system during normal maturation but also in neurodegenerative diseases and strokes.[4–7] Apoptosis also takes place within the primary neuroblastoma as the tumour cells move away from the fibrovascular stroma.[8] Positive correlations have been observed between frequency of apoptotic cells and favourable outcome of patient.[9] The mechanism behind the spontaneous regression of neuroblastoma is unknown, but one hypothesis suggests it was due to apoptosis.[10]

Apoptosis Signal-regulating Kinase 1 (ASK1) is a Mitogen Activated Protein Kinase Kinase Kinase (MAPKKK), which is typically induced by TNF-α and activates the SEK1-JNK and MKK3/MKK6-p38 signalling cascades.[11] These two pathways are activated by environmental stress, such as UV radiation and osmotic shock as well as the pro-inflammatory cytokines, tumour necrosis factor (TNF) and interleukin-1 (IL1).[12,13] ASK1 is also linked to the CD95 signalling pathway through the binding and activation of the DAXX protein.[14] Since many chemotherapeutic drugs activate this pathway, ASK1 has a central role in cytotoxicity caused by chemotherapy. Microtubules-interfering agents are used in the treatment of neuroblastoma, and it has been reported that ASK1 is involved in the signal transduction pathways initiated by microtubule interfering agents such as paclitaxel.[15,16] This antineoplastic agent promotes the in vitro assembly of microtubules, stabilising the formed polymers against depolymerisation.[17] The formation of a rigid microtubular network prevents cell division, causing block in mitosis in the G2/M phase.[18]

ASK1 induces apoptosis in a variety of cells such as mink lung epithelial cells.[11] In cells of the nervous system, rat primary sympathetic neurones and NGF-treated rat pheochromocytoma, PC12 cells have been reported to undergo apoptosis by overexpression of ASK1.[19] In contrast, Takeda et al.[20] found that moderate expression of ASK1 induces differentiation and survival of naïve PC12 cells.

The aim of this study was to investigate whether ASK1 induces apoptosis or differentiation in neuroblastoma cells and to analyze the activation of its downstream signalling pathways. We express the constitutively active mutant of ASK1, which has an N-terminal deletion (ASK1-ΔN)[21] in four different neuroblastoma cell lines, two of murine and two of human origin. We also investigate the effect of the combination of ASK1-ΔN and paclitaxel.

Results

The effect of ASK1-ΔN in C1300, NXS2, SH-SY5Y and IMR-32 cells

The effect of ASK1 in neuroblastoma cells was investigated by infecting four different cell lines, C1300, NXS2, SH-SY5Y and IMR-32 with recombinant adenoviruses encoding for the wild-type ASK1 (AdASK1-WT), the constitutively active mutant of ASK1 (AdASK1-ΔN), the dominant negative ASK1 (AdASK1-K709R) or β-galactosi-

dase (β-gal). The growth patterns of the four cell lines were monitored by [3H]thymidine incorporation during the course of 3 days (Figure 1). When the cells were grown in medium containing 10% serum, the growth of C1300 and SH-SY5Y cells was almost totally inhibited when transduced with AdASK1-ΔN at an m.o.i. of 100. While ASK1-K709R, ASK1-WT or β-gal at the same m.o.i. did not affect the growth during 3 days. The [3H]thymidine incorporation was suppressed already 24 h after infection by AdASK1-ΔN. NXS2 and IMR-32 showed growth patterns similar to the controls, and therefore they were not as sensitive as C1300 and SH-SY5Y cells to ASK1-ΔN mediated cell growth inhibition. To investigate the dose response of the four different cell lines to ASK1-ΔN, [3H]thymidine incorporation was performed at 2 and 3 days after infection in medium supplemented with 1% serum. This revealed that C1300 and SH-SY5Y are more sensitive than NXS2 and IMR-32 to the growth inhibitory effect of ASK1-ΔN, and the inhibition occurred in a dose dependent manner. We could also see that NXS2 was weakly inhibited when medium containing 1% serum was used (Figure 2).
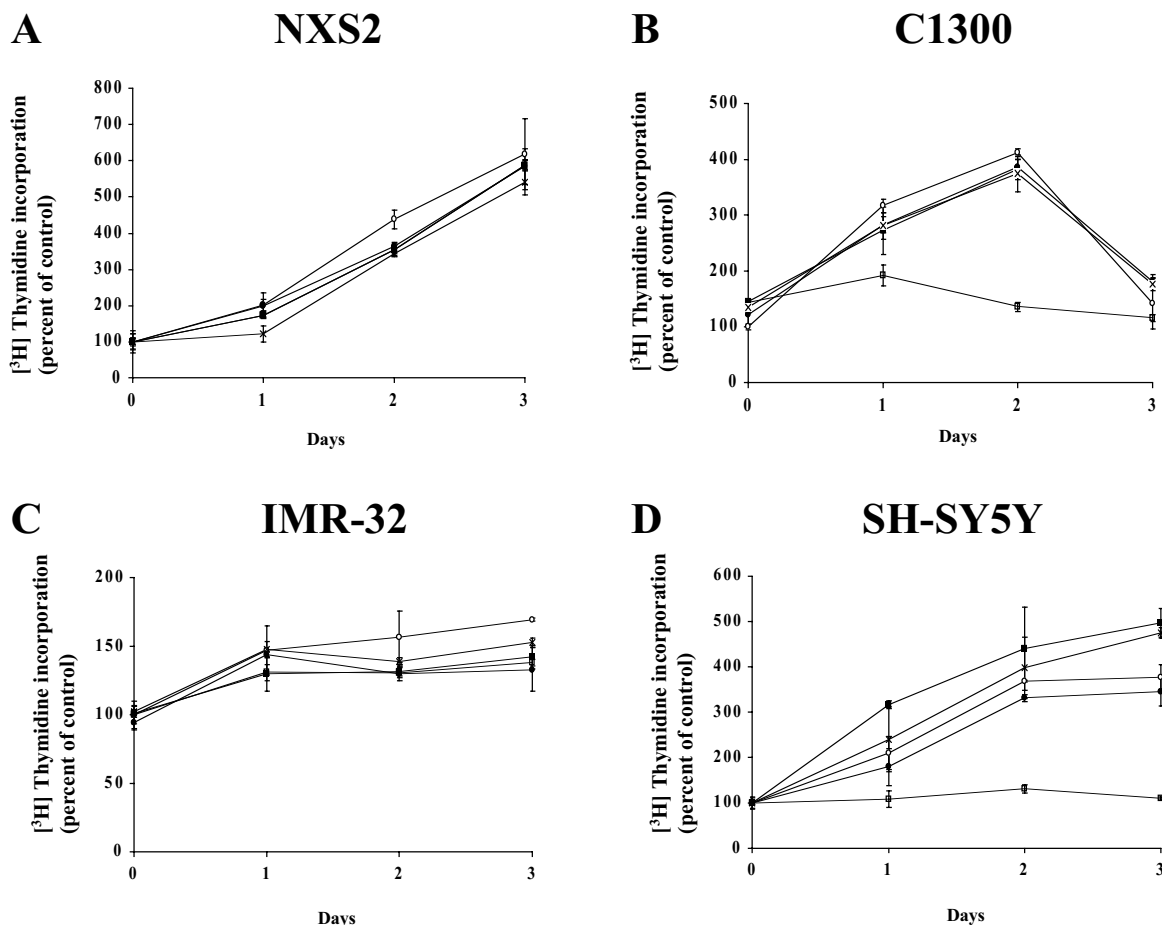

Figure 1 Time course of ASK1-ΔN dependent cell growth in different neuroblastoma cell lines. The cells were infected on day 0 with adenovirus vectors expressing β-galactosidase (●), or ASK1: wild-type, ASK1 -WT (×), constitutively active, ASK1-ΔN (□), kinase negative. ASK1-K709R (■), and uninfected (○). [3H]Thymidine incorporation was determined in medium containing 10% serum. Data shown are per cents of control that was set at the value of each cell line on the day of infection, indicated as means of triplicate values ± S.D. (A) and (B) the mouse neuroblastoma cell lines NXS2 and C1300. (C) and (D) the human cell lines IMR-32 and SH-SY5Y
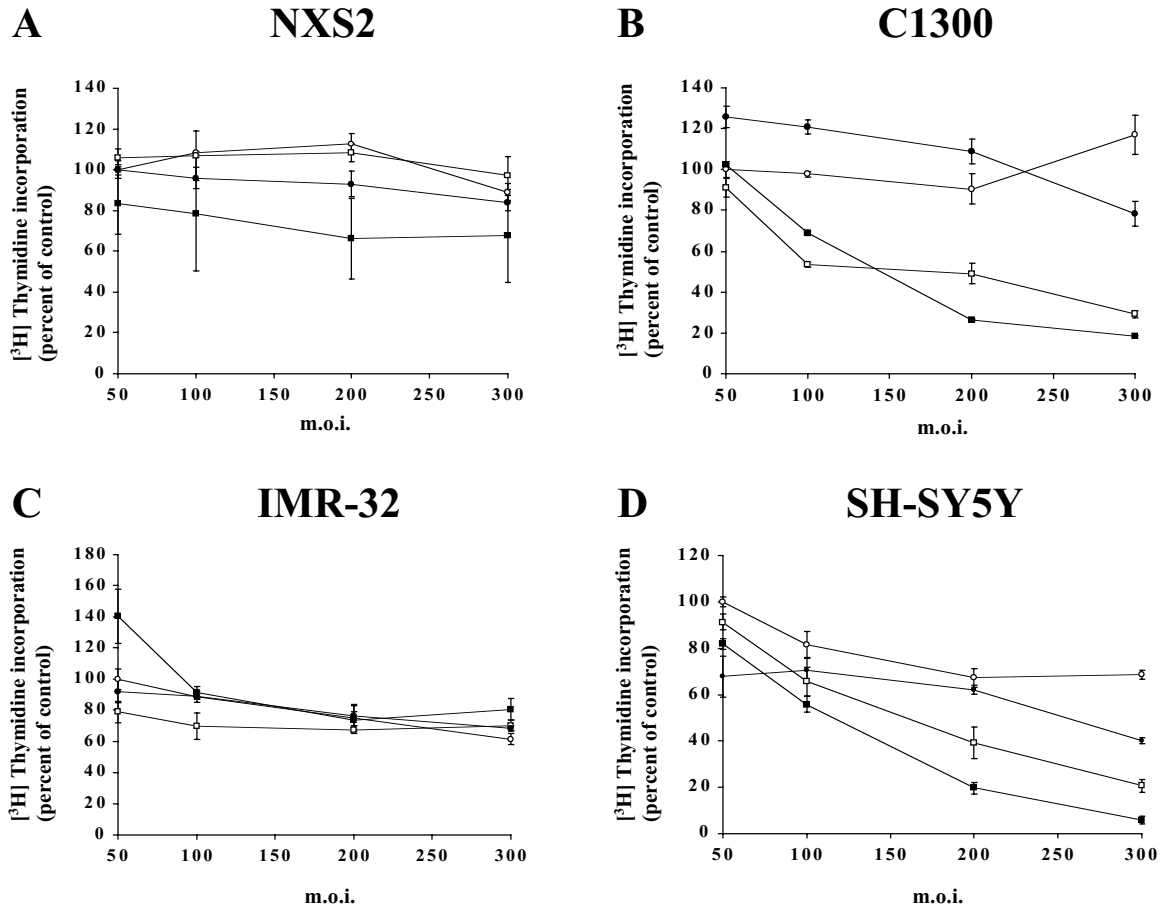
A NXS2

B C1300

C IMR-32

D SH-SY5Y

Figure 2 Dose dependent growth due to AdASK1-ΔN monitored by [³H]thymidine incorporation. (**A**) and (**B**) NXS2 and C1300. (**C**) and (**D**) IMR-32 and SH-SY5Y. ASK1-K709R (○) and ASK1-ΔN (□) 2 days after infection, ASK1-K709R (●) and ASK1-ΔN (■) 3 days after infection at an m.o.i. of 50–300. Data shown are per cents of control that was set at the value of each cell line on the day of infection, means of triplicate values ± S.D.

ASK1-ΔN promotes apoptosis in neuroblastoma cells

To establish the cause of the ASK1-ΔN induced growth inhibition in C1300 and SH-SY5Y cells, a Cell Death Detection ELISA[PLUS] (CDD) assay was performed (Figure 3A). This assay quantitatively measures the level of nucleosomes released in the cytoplasm by using antibodies against DNA and histones. There was a 3.4-fold higher level of apoptosis in SH-SY5Y cells infected with AdASK1-ΔN compared to cells infected with AdASK1-K709R. The level of apoptosis in C1300 was only 1.3-fold higher. The assay was performed on day 2 after infection in C1300 cells due to high background on day 3. When the results from the [³H]thymidine incorporation and the CDD assay were compared, a low level of apoptosis could be found in C1300 cells in the CDD assay. To verify the results of the CDD assay, FACS analysis for annexin V and propidium Iodide staining was performed. Annexin V has a high affinity for phosphatidylserine that becomes exposed on the external surface of cells during apoptosis. Compared with AdASK-K709R-infected cells, AdASK-DN-infected cells displayed an increased annexin V positive population in both SH-SY5Y and C1300 (Figure 3B). In the case of C1300, the majority of cells remained

unaffected by ASK1-ΔN and only a small population underwent apoptosis. These results from FACS analysis are concordant with those from CDD assay. Microscopic examination revealed that cells expressing ASK1-ΔN, but not the control ASK1-K709R, underwent cell death with morphological changes characteristic of apoptosis, such as nuclear condensation and membrane blebbing in C1300 and SH-SY5Y cells but not in NXS2 and IMR-32 cells (Figure 4).

ASK1-ΔN induces phosphorylation of p38 MAP kinase and c-Jun terminal kinase (JNK) in the two sensitive cell lines SH-SY5Y and C1300

ASK1 has been shown to phosphorylate the stress activated SEK1-JNK and MKK3/MKK6-p38 signalling cascades in COS cells. In this study, immunoblotting was performed to investigate the activation of these two signalling pathways in neuroblastoma cells over-expressing ASK1. The cells were harvested 24 h after infection, and immunoblotting of the lysates was performed using antibodies that specifically recognise phosphorylated p38 MAP kinase (p-p38) and phosphorylated SAPK/JNK (p-JNK) (Figure 5). The results suggest an increased phosphorylation and therefore activation of p38 in the two ASK1-sensitive cell lines, C1300 and
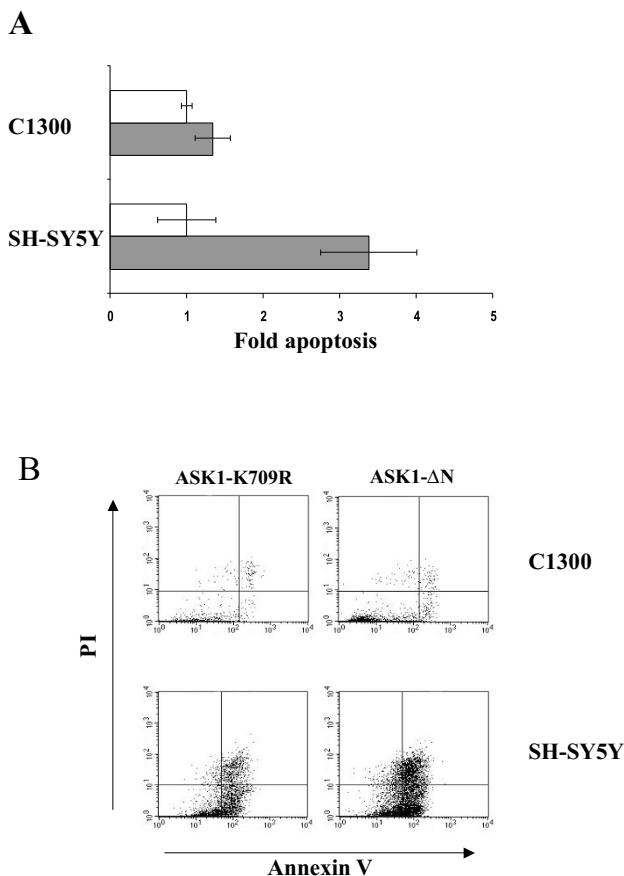
A

B

Figure 3 (**A**) Comparison of the levels of apoptosis induced by AdASK1-ΔN (grey bars) and AdASK1-K709R (white bars) measured by Cell Death Detection ELISA[PLUS]. SH-SY5Y cells were cultured in medium containing 1% for 3 days and C1300 cells for 2 days after infection at an m.o.i. of 300. Data shown are means of triplicate values ± S.D. (**B**) FACS analysis of annexin V and propidium iodide staining of ASK1-ΔN induced apoptosis in SH-SY5Y and C1300. Compared with AdASK1-K709 infected cells, AdASK1-ΔN increased in the annexin V-positive population in SH-SY5Y and C1300, although the positive population in C1300 is small. C1300 and SH-SY5Y were infected with AdASK1-K709 or AdASK1-ΔN at an m.o.i. of 300 and cultured in medium containing 1% for 2 and 3 days, respectively

SH-SY5Y, when compared with the same cells expressing the kinase negative mutant, ASK1-K709R. NXS2 showed only a marginal activation of p38 and we were not able to detect any activation in IMR-32 cells. In contrast to p38, the level of JNK activation was only slightly increased in one of the cell lines, C1300. To confirm that the cell lines had intact signalling cascades leading to p38 and JNK activation and to verify that the antibodies would detect the phosphorylated form of the antigens, lysate from cells treated with 0.5 M sorbitol for 15 min was used as a positive control.

The p38 MAPK inhibitor, SB203580, protects the cells from ASK1-ΔN mediated apoptosis

Western blot analysis suggested that ASK1-ΔN induced apoptosis in SH-SY5Y and C1300 cells through the p38 MAPK pathway. Therefore, we examined the occurrence of DNA fragmentation, and the requirement of p38 for AdASK1-

ΔN mediated apoptosis in C1300 and SH-SY5Y cells by using the p38 MAPK specific inhibitor, SB203580 (Figure 6). We first analyzed the expression of ASK1-ΔN to exclude the possibility that the presence of SB203580 decreased the expression. Equal amount of proteins was applied to Western blot and probed with the HRP conjugated anti-HA antibody (3F10), showing almost equal level of expression. We next analyzed whether the treatment of cells with SB203580 inhibited the DNA fragmentation. After 48 and 60 h, DNA laddering was clearly observed in the SH-SY5Y cells infected with AdASK1-ΔN (Figure 6A), which markedly decreased when the cells were treated with SB203580 (100 nM). In C1300 cells, DNA fragmentation was observed at 16 and 20 h after infection and was also suppressed by the presence of SB203580 (800 nM) (Figure 6B). In this experiment we used 10% FBS containing medium for SH-SY5Y cells since SB203580 itself was highly cytotoxic in a low serum condition as seen in [³H]thymidine incorporation (data not shown). Lower concentration of SB203580 was also chosen for that reason. Earlier time points for C1300 were analyzed due to high background at later stages.

ASK1-ΔN combined with paclitaxel increases cell death

The two murine cell lines NXS2 and C1300 were further analyzed for the combined effect of ASK1-ΔN and paclitaxel. The cells were infected with AdASK1-ΔN or Adβ-gal at an m.o.i. of 100, and 16 h after infection, paclitaxel was added to a final concentration of 0.01 μM (Figure 7). The cell growth was measured by [³H]thymidine incorporation after further 8, 24 and 48 h. Western blot analysis had earlier shown that ASK1-ΔN was expressed at 16 h post infection (data not shown). At the same time period of exposure, NXS2 cells were more sensitive to paclitaxel than C1300 cells and there was clearly an increased killing in both cell lines when paclitaxel was added to the ASK1-ΔN expressing cells.

Discussion

The primary purpose of this study was to investigate whether overexpression of ASK1 would induce apoptosis in neuroblastoma cells. In order to investigate the effects of ASK1-ΔN on cellular growth or viability, we measured [³H]thymidine incorporation. ASK1-ΔN dependent growth inhibition was observed in SH-SY5Y and C1300. NXS2 also showed a small tendency to growth depression. It is difficult to directly compare the cell lines to each other since there are differences in doubling time and in adenovirus infectivity between the cells, although the infection by the recombinant adenoviruses at an m.o.i. of 300 nearly transduced 100% of the cells in each cell line when estimated by β-gal expression. Otherwise, differences in the expression level of adenovirus transduced genes in these cell lines may play critical roles in their responses. The expression level of ASK1-ΔN and ASK1-K709R was comparable when estimated by Western blots at 24 h after infection.

In this study, we show that ASK1-ΔN promotes cell death due to apoptosis rather than differentiation in SH-SY5Y and C1300 cells. Immunoblotting and enzymatic
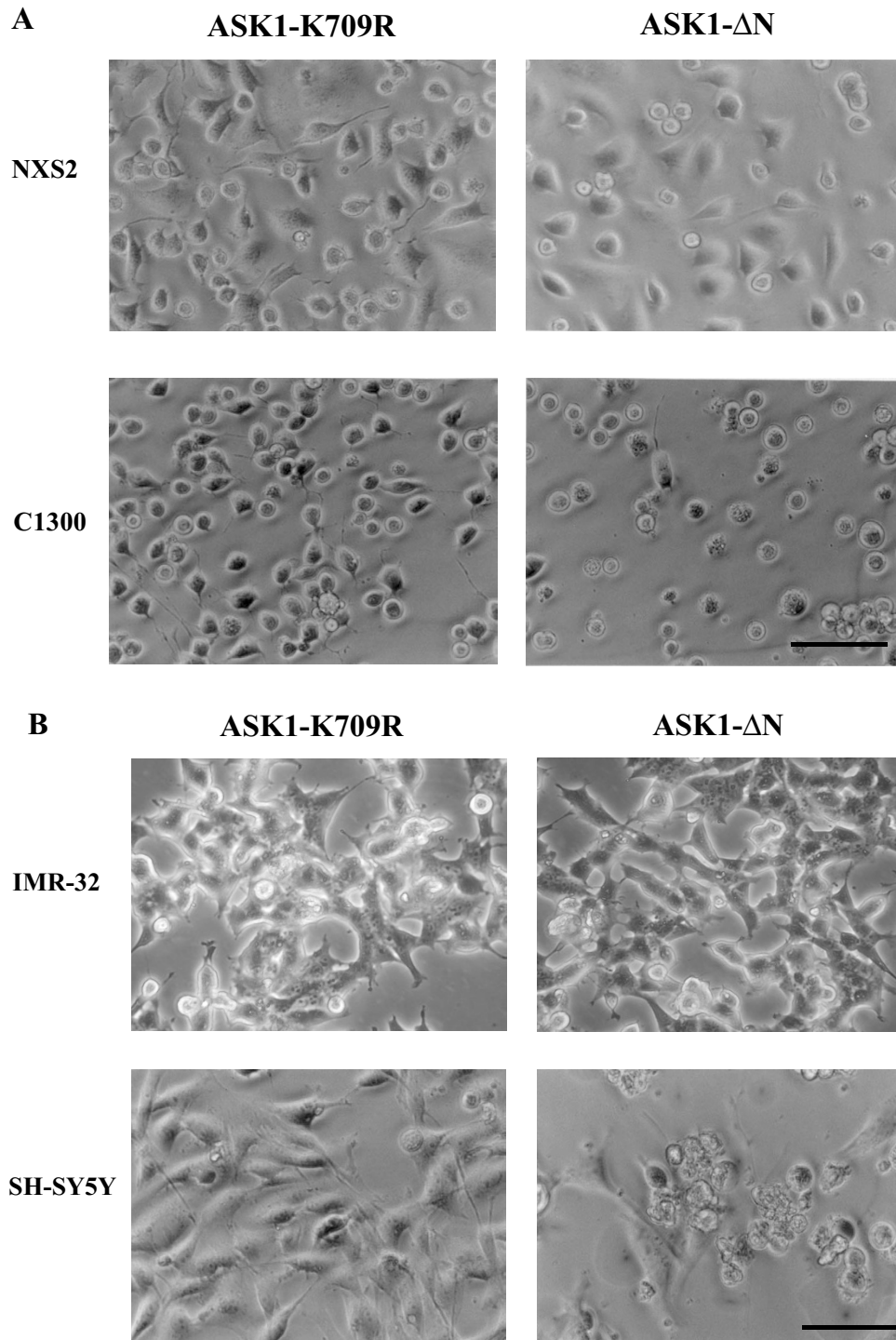
A

ASK1-K709R   ASK1-ΔN

NXS2

C1300

B

ASK1-K709R   ASK1-ΔN

IMR-32

SH-SY5Y

Figure 4 Phase-contrast micrographs showing the morphology of (**A**) NXS2 and C1300, (**B**) IMR-32 and SH-SY5Y, infected at an m.o.i. of 300 with adenovirus encoding either ASK1-K709R or ASK1-ΔN. At the point of infection the medium was changed from that containing 10% serum to 1% serum and then cultured for 3 days. Scale bar: 10 μm

analysis showed the sign of Caspase 3 activation in both of these cell lines. In the case of C1300, displaying a weak apoptotic signal in both CDD assay and annexin V analysis, we conclude that the majority of the cell population goes into cell cycle arrest, while the remaining cells go into apoptosis. C1300 has a shorter doubling time than SH-SY5Y, resulting in a rapid generation of new cell population with diluted ASK1-ΔN expression since adenovirus was used for the gene transfer. In fact, the level of phosphorylated p38 in C1300 began to decrease already
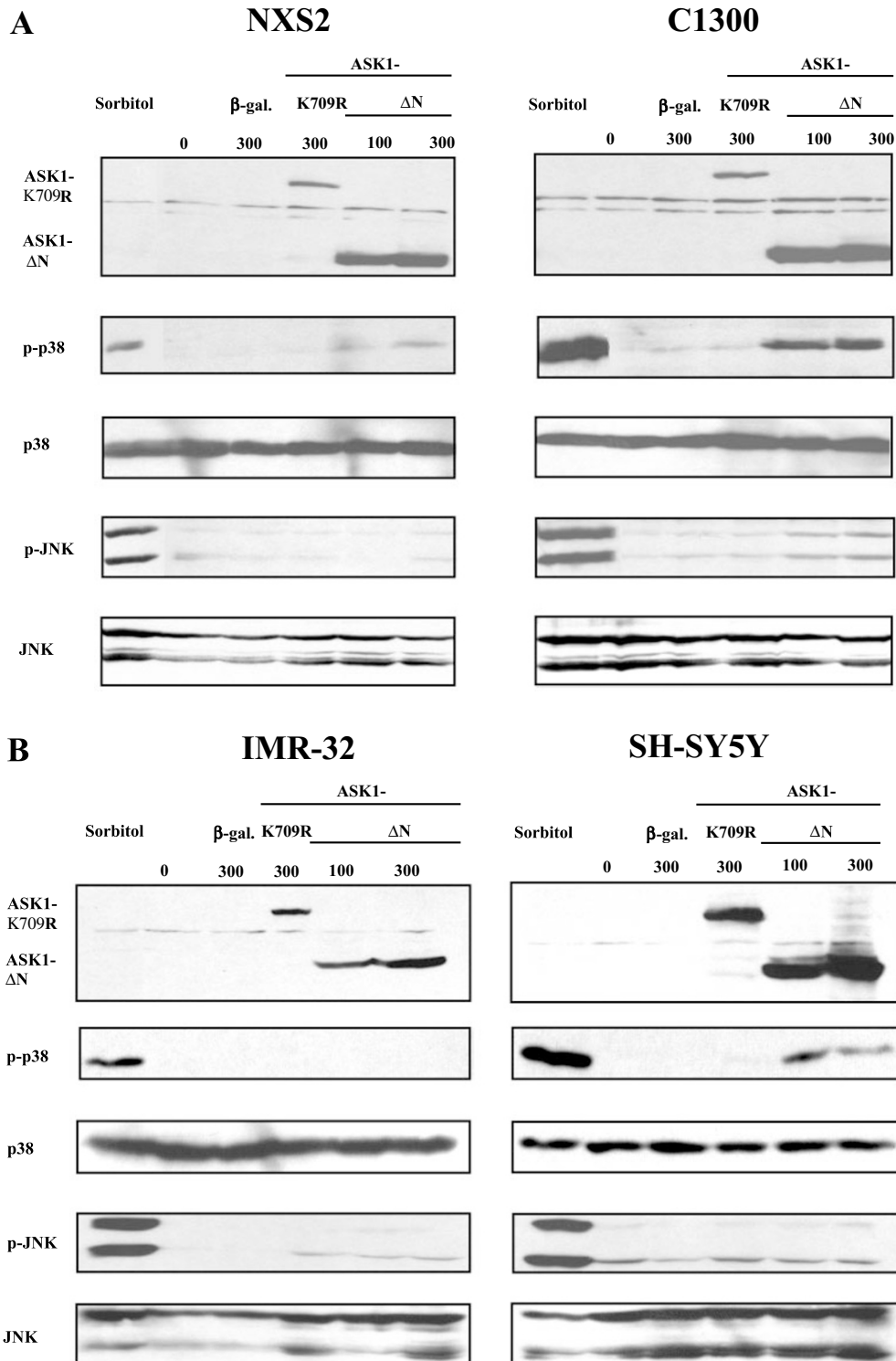
A

NXS2

C1300

B

IMR-32

SH-SY5Y

Figure 5 Immunoblotting of whole lysates from cells over-expressing β-gal, ASK1KM, and ASK1-ΔN infected at a m.o.i. of 100–300. (**A**) NXS2 and C1300. (**B**) IMR-32 and SH-SY5Y. Antibodies against the HA tagged ASK1-ΔN and ASK1-K709R, phosphorylated p38 MAP kinase (p-p38) and c-Jun kinase (p-JNK), total p38 MAP kinase (p38) and c-Jun kinase (JNK) were used. As a positive control, the lysate of the cells exposed to 0.5 M sorbitol for 15 min was used

on day 2 compared with the level seen on day 1. This agrees with the recent report demonstrating the importance of sustained activation of p38 for apoptosis.[22]

It is known that serum withdrawal induces apoptosis, and it has also been shown that 1% serum increases the ASK1 activity in Mv1Lu cells.[11] Therefore, most experi-
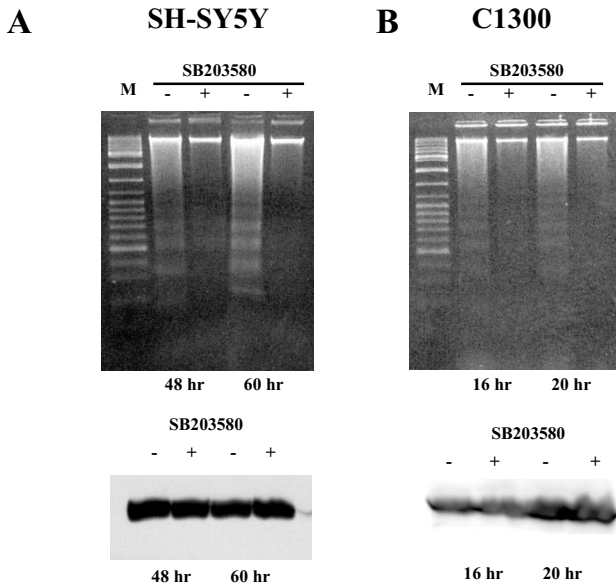
A SH-SY5Y

B C1300

Figure 6 Upper panels: The p38 MAPK specific inhibitor, SB203580, protected the ASK1 sensitive cell lines SH-SY5Y (**A**) and C1300 (**B**) from apoptosis. DNA fragmentation was observed 48 h after infection of AdASK1-ΔN in SH-SY5Y and increased after 60 h. C1300 cells showed DNA-laddering at 16 and 20 h after infection. In both cases, DNA fragmentation was reduced in the presence of SB203580 (100 nM for SH-SY5Y cells and 800 nM for C1300 cells). Lower panels: The presence of SB203580 did not affect the expression of ASK1-ΔN in SH-SY5Y (**A**) and C1300 (**B**) examined by immunoblotting using a HRP conjugated anti-HA antibody

ments were conducted in medium containing 1% serum. We could not detect any clear morphological sign of differentiation by ASK1 in any of those cell lines, in contrast to PC12 cells that differentiated in the same condition.[20] The PC12 differentiation seems to occur following p38 activation. However, in the cell lines examined herein, p38 activation was associated with the induction of cell death. The non-responding cell lines, NXS2 and IMR32, contained much lower expression levels of phosphorylated p38. We could detect a small difference in the phosphorylated JNK that was also increased in one of the sensitive cell lines, C1300. In the two murine cell lines we analyzed, NXS2 is more differentiated than C1300 since it is a hybrid of C1300 and sympathetic ganglion cells. However, NXS2 does not respond to ASK1-induced apoptosis whereas C1300 does, in contrast to PC12, which upon differentiation by NGF preferentially goes into apoptosis. The reason for this difference is not clear, but it is possible that interactions between various intracellular signalling molecules affect responses of cells to ASK1. In fact, several factors have been reported to regulate and interact with the ASK1 signalling pathway. Thus, thioredoxin binds directly to the N-terminal part of ASK1 under reducing conditions, which however cannot be involved in this study.[21] The ASK1 activity is also inhibited by cytoplasmic p21[cip1/WAF1] that forms a complex with ASK1[23] and by the 14-3-3 protein that binds specifically to Ser-967 of ASK1.[24] Cells in various conditions would therefore respond differently, depending on a balance between the activated pathways within the cells.
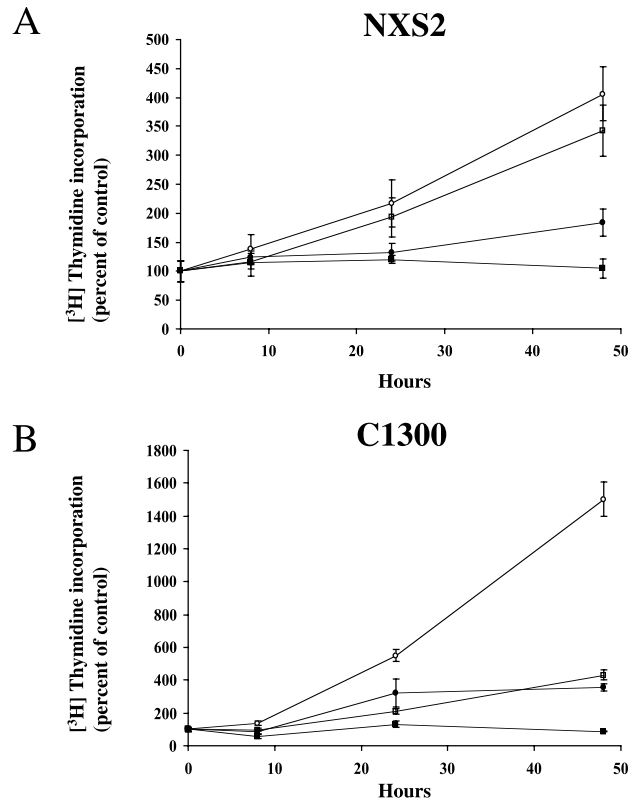

A NXS2

B C1300

Figure 7 [³H]Thymidine incorporation, 8, 24, 48 h following addition of 0.01 μM paclitaxel. Data shown are per cents of controls that were set at the value of each cell line at the time of addition of paclitaxel. (**A**) NXS2 cells. (**B**) C1300 cells. Adβ-gal+DMSO (○), Adβ-gal+paclitaxel (●), AdASK1-ΔN+DMSO (□), AdASK1-ΔN+paclitaxel (■). Means of triplicate values; bars ± S.D.

It has been shown that MYCN amplification sensitises neuroblastoma to apoptosis following their exposure to interferon γ[25] or cytotoxic drugs.[26] In this aspect, it is interesting that ASK1 enhances the protein stability of c-Myc through phosphorylation at Ser-62 and Ser-71, which is required for c-Myc-dependent apoptosis by ASK1-signalling.[27] However, MYCN is a prognostic marker for neuroblastoma with poor outcome, and despite high MYCN expression many neuroblastoma are resistant to chemotherapy. The reason for this is not clear, but it is possible that apoptosis pathways in neuroblastoma might be impaired. Our results showed that SH-SY5Y that has no MYCN gene amplification[28] was sensitive to ASK1-induces apoptosis, whereas IMR-32 that has amplification[29,30] did not show any response at all. We were not able to detect any activation of p38 MAPK in IMR-32 cells although the same kinase could be activated by osmotic shock given by sorbitol.

The plasma concentration of paclitaxel in patients under the treatment is 0.21–13.0 μM.[31] In our experiments, when 10 nM paclitaxel in combination with ASK1 was used, we were able to show an increased cytotoxic effect compared with the effects obtained by either one of these molecules. The cytotoxic effect of paclitaxel is considered to be mainly due to the phosphorylation of JNK in addition to the direct activation of ASK1.[15] It is possible that the combination of

both stress-activated kinases activates a large number of target molecules important for apoptosis. Thus, the effect of the microtubule-interfering agents used in the clinic can be potentiated by the combination with ASK1 gene transfer, which might be a possible alternative treatment. It would be valuable to investigate whether there exist proteins that counteract the ASK1-p38 pathway in certain neuroblastoma cells. The search for this mechanism is under way in our laboratory.

Materials and Methods

Cells and cell culture

Dr. RA Reisfeld (La Jolla) provided the mouse neuroblastoma cell lines, C1300 and NXS2. NXS2 is a hybrid of C1300 and sympathetic ganglion cells.[32] Dr. S Påhlman (Malmö) provided the two human cell lines, SH-SY5Y and IMR-32. All cell lines were maintained routinely in medium supplemented with 10% foetal bovine serum (FBS), penicillin 100 IU/ml and streptomycin 100 μg/ml in a 5% CO_2 atmosphere at 37°C. The medium used was either Dulbecco's modified Eagle's medium, DMEM (NXS2, SH-SY5Y), or RPMI 1640 (C1300, IMR-32).

Adenovirus vectors

The recombinant adenoviruses encoding for β-gal (Adβ-gal), the HA-tagged wild-type of ASK1 (AdASK-WT), the kinase negative ASK1 (AdASK1-K709R), and the constitutively active ASK1 (AdASK1-ΔN), were described previously.[21,33,34] The adenovirus was propagated in 293 cells to produce high titer stocks. Cells were infected for 3–4 days, harvested and lysed by five cycles of freeze-/thawing. The lysate was centrifuged at 3000 r.p.m. for 20 min at 4°C and the stocks were kept at −70°C.

ASK1-ΔN-dependent inhibition of [^{3}H]thymidine incorporation

Cells were seeded at a density of 1.5×10^4 cells per well in 24-well culture plates (Techno Plastic Products) in medium containing 10% serum and infected on the next day with recombinant adenovirus at a multiplicity of infection (m.o.i.) of 50–300. At the indicated time points the cells were incubated with 0.6 μCi [^{3}H]thymidine per ml medium for 4 h, and precipitated with 5% TCA at 4°C. The precipitate was then dissolved in 1 M NaOH, neutralised with 1 M HCl, and the ^{3}H incorporation into the DNA was determined. The cells were washed once and the medium was changed to contain 1% serum at the point of infection in the following experiments: (a) Dose response of cells expressing ASK1-ΔN (Figure 2); (b) The combined effect of ASK1 - ΔN and paclitaxel (Sigma) (Figure 7). Paclitaxel was dissolved in DMSO to a concentration of 10 mM and then added to the cells to a final concentration of 0.01 μM at 16 h after the NXS2 and C1300 cells were infected with Adβ-gal and AdASK1-ΔN at an m.o.i. of 100.

Apoptosis assay

A total of 3×10^3 cells were seeded in 0.2 ml medium in each well in a microtiter plate. On the next day the medium was changed to 1% FBS and the cells were infected with AdASK1-ΔN and AdASK1-K709R at

an m.o.i. of 300. Apoptosis was assayed using the Cell Death Detection ELISA^{PLUS} assay (Roche) according to the vendor's instruction. SH-SY5Y was assayed 3 days and C1300 2 days after infection.

FACS analysis

Annexin V staining was performed using the Annexin-V-FLURO staining kit (Roche), according to manufacturer's instructions. In short, 5×10^4 cells were infected with AdASK1-K709R or AdASK1-ΔN at an m.o.i. of 300 in medium containing 1% serum. The cells were harvested by trypsin treatment, washed twice with PBS and then resuspended in 100 μl binding buffer containing annexin V-FITC and propidum iodide (PI). After 15 min incubation, the samples were diluted with 400 μl binding buffer and the fluorescence emitted by the cells (1×10^4 cells/sample) was analyzed using a FACS Calibur flow cytometer (Becton Dickinson). SH-SY5Y was assayed 3 days and C1300 2 days after infection.

Western blot analysis

Equal numbers of cells from each cell line were seeded in medium containing 10% serum, and on the next day they were washed and medium containing 1% serum was added at the same time as the cells were infected with recombinant adenovirus. Twenty-four hours after infection the cells were washed with $1 \times$ PBS and lysed in buffer containing 150 mM NaCl, 50 mM Tris-HCl at pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride and 50 kIU/ml Trasylol. Cell debris was removed by centrifugation and the protein concentration in each lysate was determined by using DC Protein Assay (Bio-Rad). The same amount of protein, 80 μg, was separated on 10% SDS–polyacrylamide gel electrophoresis and then electroblotted onto PVDF membrane by semi-dry transfer (Bio-Rad). After blocking for 1 h with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) the membranes were probed with the primary antibodies. The primary antibodies used were phospho-specific rabbit polyclonal antibodies to p38 MAP kinase (Thr-180/Tyr-182), SAPK/JNK (Thr-183/Tyr-185) as well as rabbit polyclonal antibodies to p38 MAP kinase and JNK, and the secondary antibody was anti-rabbit IgG conjugated with horse-radish peroxidase (HRP; New England Bio-Labs). We also used the HRP-conjugated rat monoclonal antibody (3F10, Roche) to detect the haemagglutinin (HA)-tagged ASK1-K709R and ASK1-ΔN. The signals were detected by enhanced chemiluminescence (ECL; New England Bio-Labs).

DNA fragmentation analysis

Approximately 2×10^6 of SH-SY5Y and C1300 cells were seeded in 10 cm dishes and infected with AdASK1-ΔN. Following the infection, SH-SY5Y cells were cultured for 48 and 60 h in DMEM medium containing 10% FBS with or without 100 nM SB203580 (Calbiochem). In a low serum condition this inhibitor showed a toxic effect on this cell line. The C1300 cells were cultured for 16 and 20 h in RPMI containing 1% FBS with or without 800 nM SB203580. The cells were then washed with PBS and lysed with 400 μl of a buffer containing 20 mM Tris-HCl, 10 mM EDTA and 0.6% SDS (pH 7.5) by rotation for 20 min at room temperature. To remove large molecular weight DNA, 100 μl of 5M NaCl was added to the lysate and incubated overnight at 4°C. After centrifugation at $21\,000 \times g$ for 1 h, the supernatant was

recovered and incubated with 0.5 mg/ml ribonuclease A at 42°C for 2 h, and then with 0.2 mg/ml of proteinase K at 42°C for 1 h. The small molecular weight DNA fragments were then further purified by phenol, phenol-chloroform extraction, and ethanol precipitation. DNA laddering was analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

Acknowledgements

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