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Differential Response of Heat-Shock-Induced p38 MAPK and JNK Activity in PC12 Mutant and PC12 Parental Cells for Differentiation and Apoptosis

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Abstract

Among the 3 mitogen-activated protein kinases – ERK, p38 MAPK and JNK – JNK has been suggested to participate in apoptosis, whereas p38 MAPK is thought to be part of the differentiation response. There are many common inducers of JNK and p38 MAPK, but the mechanisms underlying the differential response to apoptosis and differentiation are poorly understood. We found that heatshock activated p38 MAPK at 3min after exposure to a temperature of 44 in stress-hypersensitive PC12m3 mutant cells, while it activated JNK at 20min after the same heat treatment. However, heat shock activated p38 MAPK 5min after heat treatment and JNK 10min after heat treatment in PC12 parental cells. The extent of phosphorylation of p38 MAPK induced by heat shock in PC12m3 cells was significantly greater than that in PC12 parental cells, and a high level of heat-shock-induced neurite outgrowth was observed only in PC12m3 cells. On the other hand, heat-shock-induced JNK activation appeared more quickly and apoptosis started earlier in PC12 parental cells. These findings indicate that short stress induces p38 MAPK and longer stress induces JNK, and that the response of these kinases to heat shock differs depending on cell type.

KEYWORDS: heat shock, neurite outgrowth, p38 MAP kinase, JNK, PC12 mutant cells

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Original Article

Differential Response of Heat-Shock-Induced p38 MAPK and JNK Activity in PC12 Mutant and PC12 Parental Cells for Differentiation and Apoptosis

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Among the 3 mitogen-activated protein kinases -- ERK, p38 MAPK and JNK -- JNK has been suggested to participate in apoptosis, whereas p38 MAPK is thought to be part of the differentiation response. There are many common inducers of JNK and p38 MAPK, but the mechanisms underlying the differential response to apoptosis and differentiation are poorly understood. We found that heatshock activated p38 MAPK at 3 min after exposure to a temperature of 44℃ in stress-hypersensitive PC12m3 mutant cells, while it activated JNK at 20 min after the same heat treatment. However, heat shock activated p38 MAPK 5 min after heat treatment and JNK 10 min after heat treatment in PC12 parental cells. The extent of phosphorylation of p38 MAPK induced by heat shock in PC12m3 cells was significantly greater than that in PC12 parental cells, and a high level of heat-shock-induced neurite outgrowth was observed only in PC12m3 cells. On the other hand, heat-shock-induced JNK activation appeared more quickly and apoptosis started earlier in PC12 parental cells. These findings indicate that short stress induces p38 MAPK and longer stress induces JNK, and that the response of these kinases to heat shock differs depending on cell type.

Key words: heat shock, neurite outgrowth, p38 MAP kinase, JNK, PC12 mutant cells

p38 MAPK and c-Jun N-terminal kinase (JNK, also called stress-activated protein kinase, SAPK), together with extracellular signal-regulated kinase (ERK), constitute the family of MAP kinases. While ERK is mainly activated by mitogenic stimuli, the JNK and p38 pathways are activated by pro-inflammatory or stressful stimuli. JNK and its substrate c-Jun have been suggested to participate in the apoptosis or degeneration of neurons *in vitro* and in the brain [1]. Apoptosis can be induced by a variety of different signals, including activation of Fas or tumor necrosis factor receptors, deprivation of growth factors, treatment with chemotherapeutic drugs, excessive DNA damage, and stresses such as heat shock and

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hyperosmotic shock [2, 3]. A damaged neuron exposed to excessive stress may be removed by apoptosis induced by JNK or other signaling systems in the brain. While there are many common inducers of JNK and p38 MAPK, p38 MAPK has a distinctly different stress response from that of JNK. Activation of p38 MAPK is thought to have various cellular functions, including those of differentiation and the nervous system [4, 5].

We obtained a variant cell line that spontaneously showed impaired NGF-induced neurite outgrowth during continuous culturing of PC12 cells. When these cells were cultured for 2 weeks under acidic conditions of Cl^-, several surviving clones appeared. Using the ring isolation procedure, 10 colonies were selected and propagated in a mass culture. Among these isolated clones, a PC12 mutant clone termed PC12m3 showed poor neurite outgrowth in spite of the normal sustained activation of ERK by NGF treatment. However, neurite outgrowth of PC12m3 cells was stimulated by various drugs, such as calcimycine, c-AMP, and steroid, in the presence of NGF [6]. Furthermore, treatment of PC12m3 cells with stressful stimuli such as heat shock, radiation, and osmotic shock in the presence of NGF had strong stimulatory effects on neurite outgrowth [5, 7].

In our previous study, we investigated the role of the p38 MAPK pathway in heat-shock-induced neurite outgrowth of PC12m3 cells [7]. When cultures of PC12m3 cells were exposed to heat stress at 44℃ for 10 min, p38 MAPK activity increased and neurite outgrowth was greatly enhanced. We found that heat shock activated both ERK and p38 MAPK in PC12 parental cells only p38 MAPK in PC12m3 cells. Furthermore, the extent of p38 MAPK phosphorylation induced by heat shock was much greater in PC12m3 cells than in PC12 parental cells. Heat-shock-induced neurite extension was inhibited by treatment with the p38 MAPK inhibitor SB203580. These findings indicate that activation of the p38 MAPK pathway is necessary for heat-shock-induced neuronal differentiation of PC12m3 cells. However, very recently, Munyappa and Das demonstrated that the specific p38 MAPK inhibitors SB202190 and SB203580 activate JNK [8]. Further experiments using multiple cell lines as well as primary cell lines and primary endothelial cells showed that treatment of cells with SB202190 and SB203580 resulted in the phosphorylation of JNK and activation of transcription factor 2 (ATF-2) as well as an increase in AP-1 DNA binding. Since SB203580 is widely used to dissect the JNK/p38 MAPK signaling pathways, and since the conclusions of Munyappa and Das are based on the inhibition of p38 MAPK by SB203580, their study provides important information for the examination of whether or not the effects observed due to the use SB203580 are consequences of p38 MAPK inhibition or JNK activation. In this study, we elucidated the mechanisms of differential response of heat-shock-induced p38 MAPK and JNK activities for differentiation and apoptosis without using specific p38 MAPK inhibitors.

Materials and Methods

Reagents and cell culture. NGF (2.5S) was purchased from Takara (Osaka, Japan). PC12 parental and PC12m3 mutant cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.35% glucose, 10% horse serum, 5% fetal bovine serum (FBS), and 100 units/ml kanamycin. All cells were grown at 37℃ in 5% CO_2.

Determination of neurite outgrowth. A single-cell suspension of PC12 parental and PC12m3 cells was obtained by trituration in DMEM. For experiments on neuritogenesis, the cells were plated in 25 cm^2 flasks at a density of $2-5\times10^5$ cells per dish of serum-containing DMEM and then exposed to a heat stress of 44℃ for 10, 20, or 30 min using a water bath. After 7 days of incubation, the lengths and numbers of neurites were measured. Cells possessing neuritis whose lengths were at least 1.5-fold greater than the diameter of the cell body were counted as previously described [8]. Each value is the mean ± S.D. for 200 cells sampled from 3 independent experiments.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL). A commercially available *in situ* death detection kit was utilized to assess DNA fragmentation. This kit was purchased from Roche (Mannheim, Germany). PC12m3 cells (5×10^5 cells/3 ml on a chamber slide) were fixed for 30 min in 10% neutral buffered formalin solution at room temperature. Endogenous peroxidase was inactivated by incubation with 0.3% hydrogen peroxide in methanol for 30 min at room

temperature and further incubated in a permeabilizing solution (0.1% solution citrate and 0.1% Triton X-100) for 2 min at 4℃. The cells were incubated with the TUNEL reaction mixture for 60 min at 37℃, followed by labeling with peroxidase-conjugated anti-goat antibody (Fab fragment) for an additional 30 min. The cells were rinsed with phosphate-buffered saline (PBS) and examined under a fluorescent microscope (model 1×70; Olympus, Tokyo, Japan).

Detection of activated p38 MAPK and JNK. p38 MAPK and JNK activities were determined as described previously [9]. Briefly, PC12 parental and PC12m3 cells were plated at a density of 1×10^6 cells /25 cm^2 in a flask of serum-containing medium and cultured for 3 days. Then the culture medium was replaced by 0.5% FBS-containing medium, and the cells were cultured for a further 48 h. PC12 parental and PC12m3 cells were then exposed to heat shock (temperature of 44℃) for 3, 5, 10, or 20 min. MAPK activity in cell lysates was then assayed. The cells were lysed in a lysing buffer. Aliquots of the lysates (10–15 μg) from each sample were fractionated on SDS-10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The blots were probed with antibodies specific for phospho-p38 MAPK (Thr 180/Tyr 182), phospho-JNK (Thr 183/Tyr 185), phospho-Hsp27 (Ser 82), phospho-cJun (Ser 73), total JNK, or total p38 MAPK (New England BioLabs; Beverly, MA, USA) at a dilution of 1: 1,000 in blocking buffer (5% nonfat dry milk) for 12 h at 4℃. The blots were probed with a secondary antibody, horseradish peroxidase-linked anti-rabbit IgG, at a dilution of 1: 2,000 in blocking buffer for 60 min at room temperature. The blots were stained for 1 min using a nucleic acid chemiluminescence reagent (LumiGLO chemiluminescent reagent, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and exposed to x-ray film.

Results

Heat-shock-induced neurite outgrowth and apoptosis in PC12m3 and PC12 parental cells. Heat-shock treatment of PC12m3 cells at 44℃ for 10 min induced neuronal differentiation, as clearly shown by the appearance of neurite outgrowth. NGF induced only minor neurite outgrowth in PC12m3 cells. PC12m3 cells showed a 6-fold higher frequency of neurite outgrowth induced by heat shock than that of nontreated control cells, which showed neuronal morphology similar to that of PC12 parental cells that had undergone NGF-induced differentiation (Figs. 1 and 2). In comparison, PC12 parental cells showed a low level of heat-shock-induced neurite outgrowth. The frequency of neurite outgrowth of PC12m3 cells induced by heat shock was approximately 8.5-fold greater than that of PC12 parental cells. The frequency of heat-shock-induced neurite outgrowth in both PC12 parental and PC12m3 cells without NGF was similar to that in NGF-treated cells [7]. On the other hand, with longer (20 or 30 min) heat treatment at 44℃, the cytotoxic effects were prominent in both PC12m3 and PC12 parental cells. Interestingly, after 10-min heat treatment at 44℃, no cytotoxic effect was observed in PC12m3 cells, but cell death had already started under the same heat conditions in PC12 parental cells (Fig. 1). One of the distinctive biochemical hallmarks of apoptotic cell death is the unique occurrence of internucleosomal DNA fragmentation. Apoptotic cells were confirmed by TUNEL staining, which is widely used to detect DNA fragmentation *in situ*. Apoptotic cell death induced by heat shock in both PC12 parental and PC12m3 cells was revealed by an increased proportion of TUNEL-positive cells (Figs. 3 and 4).

Activation of p38 MAPK and JNK induced by heat shock in PC12 cells. It has been shown that activation of the p38 MAPK pathway is required, in addition to that of the ERK pathway, for NGF-induced neurite outgrowth of PC12 cells [4]. Heat-shock-induced activation of p38 MAPK was relatively weak in PC12 parental cells, whereas strong activation of p38 MAPK was detected in PC12m3 cells, for which NGF treatment induced only minor neurite outgrowth. Time-course experiments of p38 MAPK activation by heat shock in PC12m3 cells showed that heat-shock-induced p38 MAPK activation started within 3 min after exposure to a temperature of 44℃, peaked within 5 min, and then returned to the baseline level within 20 min. On the other hand, heat-shock-induced p38 MAPK activation of PC12 parental cells was delayed, appearing at 5 min after exposure to a temperature of 44℃ and being sustained for up to 20 min. However, the starting time of heat-shock-induced JNK activation of PC12 parental cells was about 10 min earlier than that of PC12m3 cells. Heat-

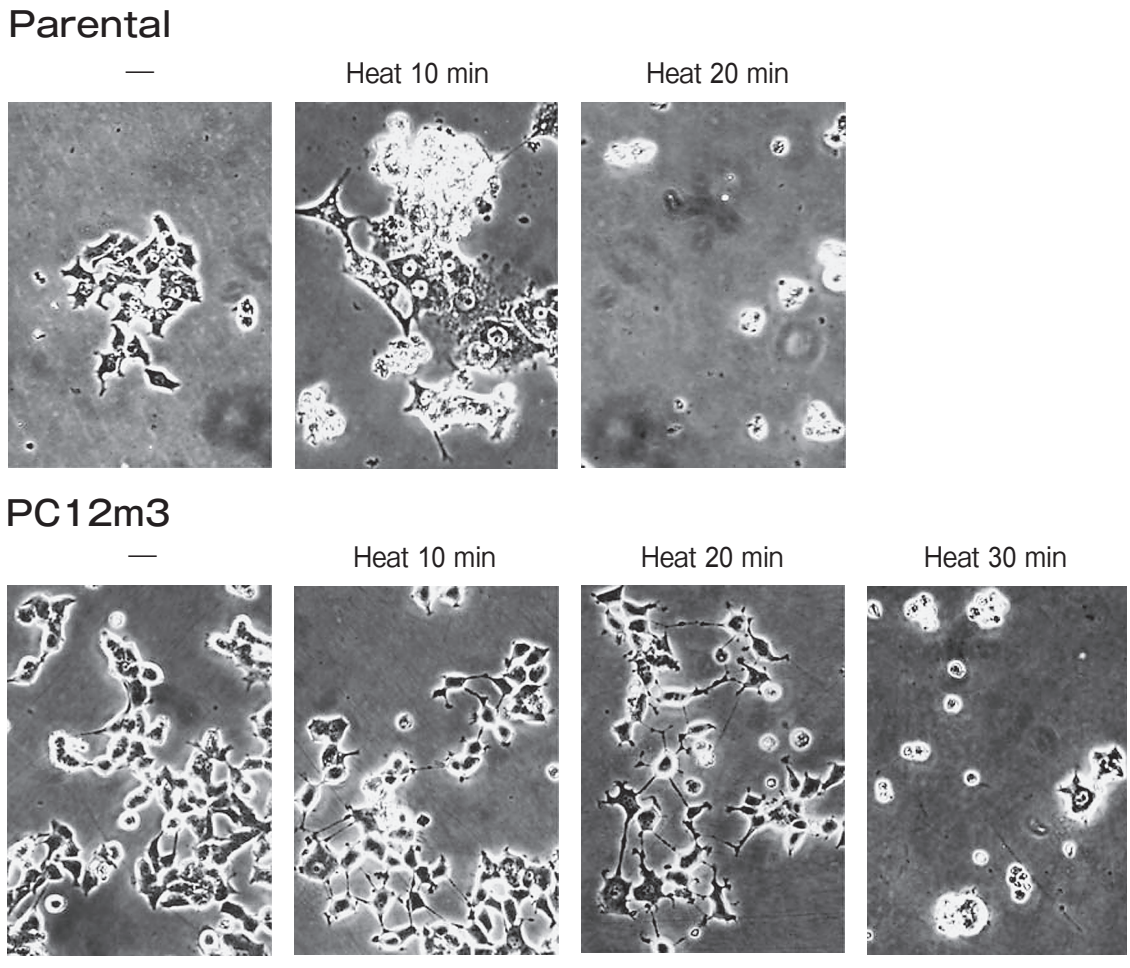

Fig. 1 Stimulation of neurite outgrowth or induction of apoptosis in PC12 cells by heat-shock treatment. PC12 parental and PC12m3 cells were exposed to a temperature of 44℃ for 10 min (Heat 10 min), 20 min (Heat 20 min), or 30 min (Heat 30 min). Phase-contrast photomicrographs of PC12 cells were taken 7 days after treatment.

shock-induced JNK activation of PC12 parental cells appeared after exposure to a temperature of 44℃ for 10 min, the same time as the appearance of cell toxicity by heat shock in PC12 parental cells. These results showed that heat-shock treatment at 44℃ for 10 min activates both p38 MAPK and JNK and has a toxic effect on PC12 parental cells, whereas heat-shock treatment at 44℃ for 10 min activates only p38 MAPK and induces differentiation of PC12m3 cells (Fig. 5).

Heat-shock caused the activation of both p38 MAPK and JNK. Since Hsp27 is phosphorylated on Ser^{82} by MAPKAPK-2, a target of p38, we next investigated p38 MAPK's involvement in this phosphorylation process. Western blot analysis showed higher levels of Hsp27 phosphorylation in PC12m3 cells than in PC12 parental cells after heat-shock treatment (Fig. 5). These results indicate a differential role for p38 MAPK in the control of Hsp27 phosphorylation between PC12 parental and PC12m3 cells. Phosphorylation of transcription factor c-Jun is a major result of JNK activation. As shown in Fig. 5B, treatment of cells with heat shock at 44℃ for 10 and 20 min resulted in the phosphorylation of c-Jun in PC12 parental cells, suggesting that JNK is activated by treatment of cells with heat shock.

Discussion

We found that heat shock activated both p38

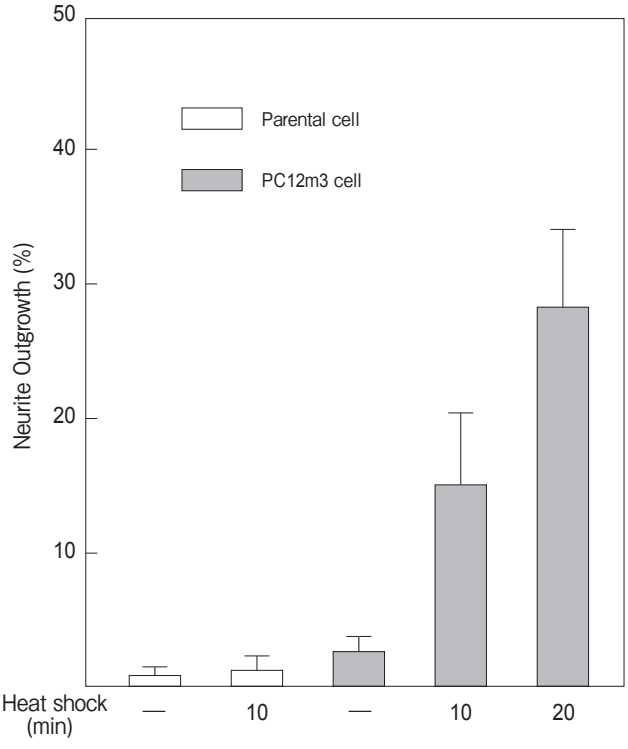

Fig. 2 Frequencies of neurite outgrowth in PC12 cells induced by heat shock. PC12 parental and PC12m3 cells were exposed to heat shock at 44℃ for 10 min and at 44℃ for 20 min. The percentage of cells with neurites was determined after 7 days. Each value is the mean ± S.D. for 200 cells sampled from 3 independent experiments.

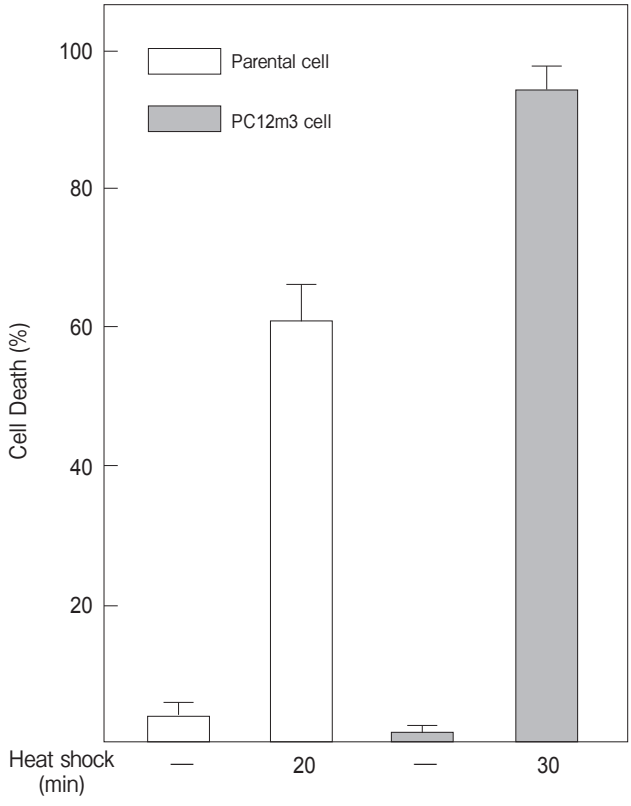

Fig. 4 Frequency of heat-shock-induced cell death in PC12 cells. PC12m3 and PC12 parental cells were exposed to heat shock at 44℃ for 20 and 30 min. The percentage of TUNEL-positive cells was determined after 2 days. Each value is the mean ± S.D. for 200 cells sampled from 3 independent experiments.

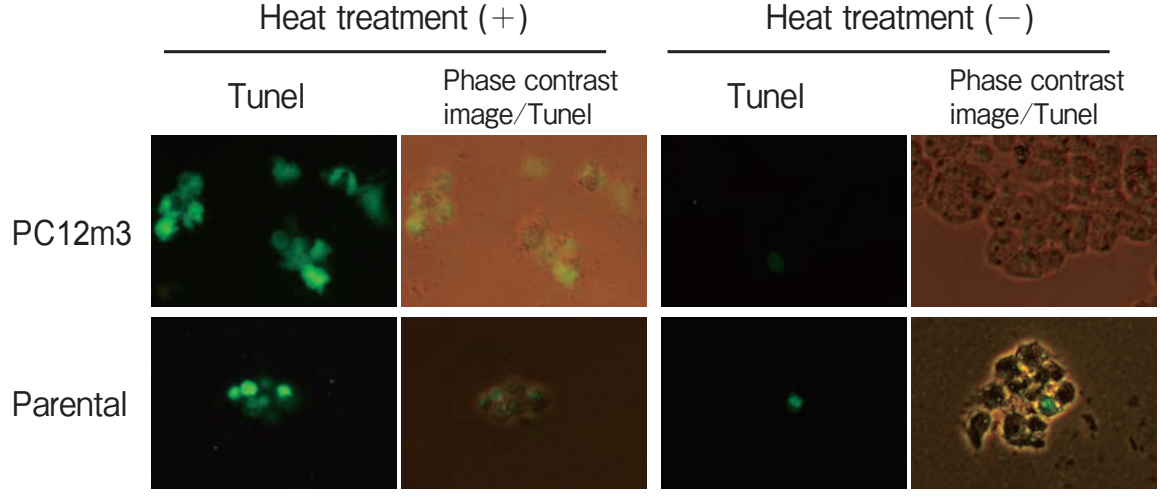

Fig. 3 Detection of heat-shock-induced cell death. PC12 parental and PC12m3 cells were exposed to heat shock at 44℃ for 20 min (Parental) or 44℃ for 30 min (PC12m3). Then the cells were cultured for 48 h. Heat-shock-induced internucleosomal DNA fragmentation was determined by TUNEL staining.

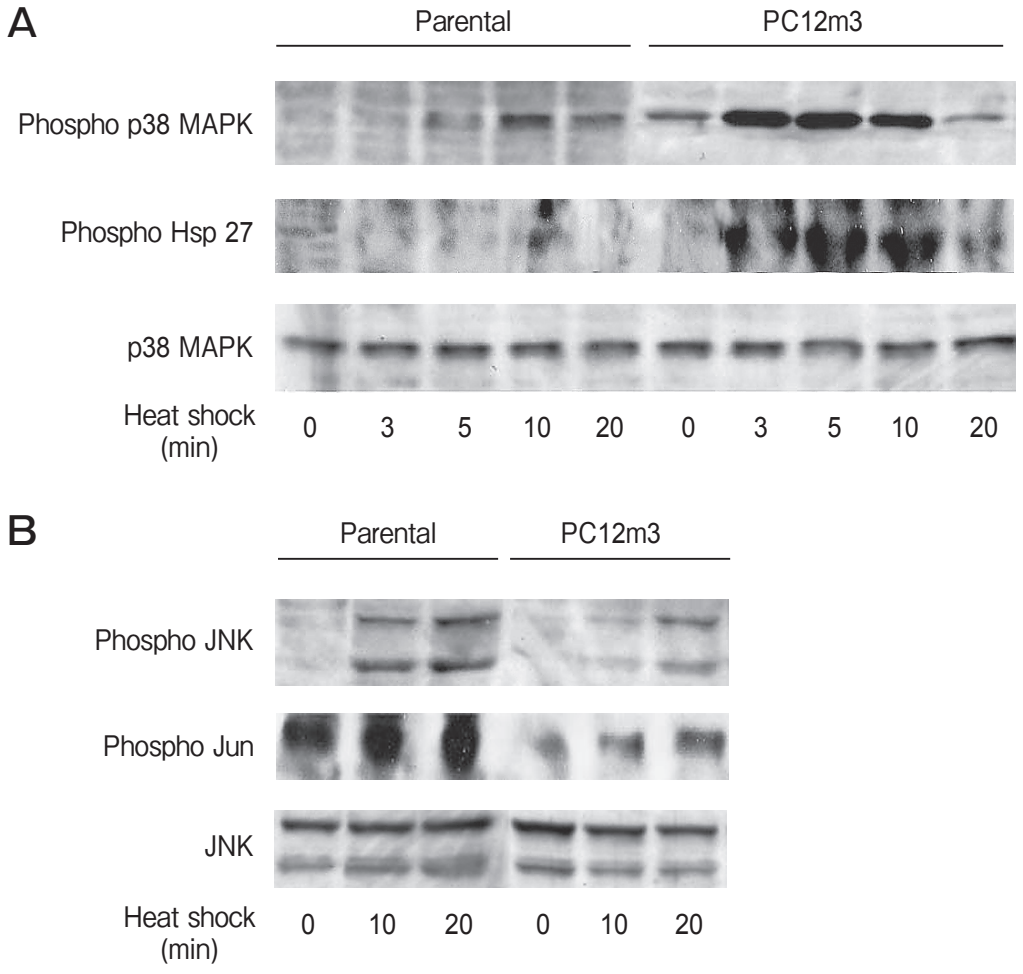

Fig. 5 Activation of p38 MAPK and JNK induced by heat shock in PC12 cells. (**A**) PC12 parental and PC12m3 cells were serum-starved and exposed to heat shock at 44℃ for 0, 3, 5, 10, and 20 min. After treatment, cells were lysed, and protein extracts were analyzed by Western blotting using antibodies specific for phospho-p38 MAPK, phospho-Hsp 27, or total p38 MAPK. (**B**) PC12 parental and PC12m3 cells were serum-starved and exposed to heat shock at 44℃ for 10 and 20 min, and extracts were subjected to Western blotting with anti-phospho-JNK, anti-phospho-cJun, or anti-total JNK antibodies.

MAPK and JNK in PC12m3 and PC12 parental cells, but the two cell lines showed differential responses to heat-shock-induced activation of p38 MAPK and JNK for differentiation and apoptosis. Heat shock stimulates p38 MAPK phosphorylation in PC12m3 cells within 3 min after exposure to a temperature of 44℃. Phosphorylation peaks within 5 min and then returns to baseline within 20 min. On the other hand, p38 MAPK phosphorylation induced by heat shock appeared at 5 min after exposure to heat shock, peaked at 10 min, and was sustained for up to 20 min in PC12 parental cells. The extent of p38 MAPK phosphorylation induced by heat shock was significantly greater in PC12m3 cells than in PC12 parental cells, and a high level of heat-shock-induced neurite outgrowth was observed only in PC12m3 cells. On the other hand, when PC12m3 cells were exposed to heat shock at 44℃ for 20 min, JNK was activated and the induction of apoptosis had started. Interestingly, heat-shock-induced JNK activation appeared after exposure at 44℃ for 10 min, the time when apoptosis was observed in PC12 parental cells. This activation time of JNK by heat shock is consistent with the induction of apoptosis by heat shock in both PC12m3 and PC12 parental cells. These data indicate that p38 MAPK is associated with the induction of differentia-

tion and that JNK may play a critical role in apoptosis.

It has been proposed that, in neurons [10] and proximal tubule cells [11], the relative extents of JNK, p38 MAPK, and ERK activation may determine cell fate, with JNK activation being associated with cell death and ERK activity being protective. JNK has been implicated in the mitochondrial death pathway [12]. Nagata and Todokoro found that brief exposure of SKT6 cells, which respond to erythropoietin (EPO) and undergo erythroid differentiation, to osmotic or heat shock induced transient activation of JNK and p38 MAPK as well as inactivation of ERK, and resulted in erythroid differentiation without EPO, whereas long exposure to these stresses induced prolonged activation/inactivation of the same kinases and caused apoptosis [13]. These results indicate that brief p38 MAPK and/or JNK activation caused erythroid differentiation without EPO, although its prolonged activation induced apoptosis. Chen *et al.* also reported that T-cell activation signals through CD28 induced rapid and transient JNK activation in Jurkat T-cells, which in turn stimulated cell growth, whereas γ irradiation or UV light caused delayed and persistent JNK activation, which led to apoptotic cell death [14]. These results indicate that the different timing and/or duration of JNK and p38 MAPK activation may alter the cell proliferation, differentiation, or apoptosis. However, our results indicate that a small permissible stress may activate p38 MAPK and induce differentiation but that excessive stress may induce apoptosis by JNK activation.

Nagata and Todokoro hypothesized that the dynamic balance between ERK and JNK-p38 MAPK may contribute to the determination of cell fate, whether cells undergo proliferation, differentiation, or apoptosis. However, our data indicate that the single p38 MAPK pathway can induce differentiation of PC12m3 cells, because in heat-shock-induced induction of neurite outgrowth of PC12m3 cells, p38 MAPK was activated by heat shock at 44℃ for 10 min, but ERK [7] and JNK were not activated under the same heat conditions (Fig. 5). Furthermore, PC12m3 cells have poor neurite outgrowth despite showing normal sustained activation of ERK by NGF treatment [7].

It has already been shown that cells respond to exposure to elevated temperature by activation of p38 MAPK and JNK as well as by increasing production of heat shock proteins such as Hsp27, Hsp70, and Akt [15, 16]. One target of p38 MAPK is MAPKAP-K2, which phosphorylates Hsp27. This, in turn, modulates both the chaperone activity and anti-apoptotic effects of Hsp27 [17]. Hsp70 protects cells from a number of apoptotic stimuli by its chaperone functions in protein folding and assembly [18, 19]. Hsp70 can also modulate stress-activated signaling through direct binding to JNK [20]. When PC12m3 cells were exposed to heat shock at 44℃ for 10 min, apoptosis was not observed, whereas cell death had already started under the same heat conditions in PC12 parental cells. These phenomena may depend on the strong activation of p38 MAPK and heat-shock proteins by heat-shock treatment in PC12m3 cells.

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