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学 位 論 文 題 目	The apoptotic volume decrease is an upstream event of MAP kinase activation during staurosporine-induced apoptosis in HeLa cells
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Apoptosis is an important cellular process, which eliminates unwanted cells during normal development or damaged cells after removal of trophic factors or exposure to toxic chemicals. In addition, anti-cancer drugs induce apoptosis in cancer cells. Apoptosis defined by biochemical and morphological changes includes cell volume decrease, caspase activation, chromatin condensation, genome DNA fragmentation, and apoptotic body formation. The apoptotic volume decrease (AVD), an early component of apoptotic cell death, is driven by osmolyte efflux resulting mainly from the activation of K^+ and Cl^- conductances. The resultant shrinkage has been used as a key discriminator between apoptosis and necrosis. Activation of the volume-sensitive outwardly rectifying anion channel (VSOR), which is involved in the regulatory volume decrease (RVD), has been indicated to cause AVD.

On the other hand, the stress-responsive MAP kinase cascade is also known to be involved in the apoptotic cell death. The MAP kinases constitute the most extensively studied cascade, which transmits extracellular stimuli to the nucleus. The MAP kinase cascade is composed of three kinases; MAP kinase (MAPK), MAP kinase kinase (MAPKK), and MAP kinase kinase kinase (MAPKKK). MAPKKK phosphorylates and thereby activates MAPKK, which in turn phosphorylates and activates MAP kinase. Apoptosis signal-regulating kinase 1 (ASK1), a 160 kDa serine/threonine protein kinase, is a member of MAPKKK family. ASK1 activates both c-Jun N-terminal kinase (JNK) and p38 MAPK through phosphorylation of several MAPKKs. ASK1 is a general mediator of apoptotic cell death in response to a variety of stimuli and especially to oxidative stress. In the present study, the author investigated the relationship between AVD and MAP kinase cascade activation during the process of staurosporine-induced apoptosis in HeLa cells.

First, He showed that in HeLa cells, caspase 3/7 activation and cell death induced by staurosporine (STS) was inhibited by application of VSOR blockers. The AVD process started immediately after treatment with 4 μM STS. When a VSOR blocker (200 μM NPPB or 100 μM phloretin) was applied together with STS, the AVD was suppressed. An increase in caspase 3/7 activity was observed 4 hr after STS treatment. Similarly, a VSOR blocker (200 μM NPPB or 100 μM phloretin) inhibited STS-induced activation of caspase 3/7. Next, He observed that VSOR blockers also suppressed STS-induced phosphorylation of p38 and JNK proteins. Activation of p38 during apoptotic stress given by addition of 4 μM STS was investigated by western blot analysis using antibodies directed against p38 and its phosphorylated form (phospho-p38). The phosphorylation level of p38 increased at ≥20 min after STS treatment. However, in the presence of a VSOR blocker (200 μM NPPB or 100 μM phloretin), STS-induced phosphorylation of p38 became less marked in comparison with that in the absence of the VSOR blocker. An increase in the JNK phosphorylation was also observed by apoptotic stimulation with 4 μM STS. A VSOR blocker suppressed phosphorylation of JNK. These data indicate that VSOR blockers inhibit MAPK phosphorylation in response to STS treatment. A p38 MAPK inhibitor, SB203580, and a JNK MAPK inhibitor, SP600125, did not inhibit AVD induced by STS, whereas these compound inhibited STS-induced p38 and JNK phosphorylation, respectively. Furthermore, treatment with

ASK1-specific siRNA showed decreases in the ASK1 mRNA level and STS-induced caspase-3/7 activation. This indicates that ASK1 is requisite for the STS-induced caspase-3/7 activation in HeLa cells. And, the STS-induced AVD in siRNA-mediated ASK1 knock-down cells was compared with that in negative control siRNA-transfected cells. Cell volume of the ASK1 knock-down cells was decreased by the STS stimulation, and the cell volume decrease was not different from that of the negative control cells. These data suggest that ASK1 activity is not necessary for the STS-induced AVD in HeLa cells. Consequently, it is concluded that the MAPK activation is a downstream event of AVD during apoptotic cell death.

If AVD is an upstream causative factor for the stress-responsive MAP kinase cascade, sustained cell shrinkage per se should activate the MAP kinase cascade and induce apoptotic cell death. To study this hypothesis, the effect of sustained cell shrinkage was examined using hypertonic stimulation (600 mOsm) in the presence of 100 μM flufenamate (FFA), which is a blocker of hypertonicity-induced cation channel (HICC) involved in the regulatory volume increase (RVI). Under the conditions, it was shown that cell volume was not restored by the RVI mechanism from the initial cell shrinkage induced by a hypertonic challenge. The hypertonic stress with FFA for 4 hr resulted in caspase-3/7 activation and decreased cell viability. Under the same conditions, activation of the stress-responsive MAP kinase cascade was then examined. The hypertonic stress together with FFA increased the p38 phosphorylation. Interestingly, the onset of phosphorylation of p38 induced by the sustained cell shrinkage was faster than that by the STS-induced AVD. This phosphorylation of p38 induced by the sustained cell shrinkage was dependent on osmolarity. The p38 phosphorylation and JNK phosphorylation were positively correlated with osmolarity. To eliminate the possibility that VSOR blockers inhibited hypertonicity-induced phosphorylation of the stress-responsive MAP kinases, the phospho-p38 level was examined under conditions in the presence of NPPB (200 μM). The hypertonicity-induced p38 phosphorylation was not inhibited by the NPPB treatment. These data indicate that the sustained cell shrinkage per se induces activation of the stress-responsive MAP kinases even without VSOR activation.

These data indicate that activation of p38 and JNK in a significant downstream event of AVD for the STS-induced apoptosis of HeLa cells. Since activation of the MAP kinases was observed under hypertonic conditions inducing sustained cell shrinkage, it is suggested that the sustained cell shrinkage is indispensable for STS-induced MAP kinase activation to cause apoptotic cell death in HeLa cells.

論文の審査結果の要旨

HeLa 細胞等にスタウロスポリン刺激を与えると、細胞死が誘導される。その過程において、アポトーシス性細胞容積減少と、MAP キナーゼ系の活性化が起こることが知られているが、ふたつの現象のどちらがより上流に位置するかは、興味深い問題であるにも関わらず、未だ明らかにされていない。本論文に於いて、申請者長谷川裕一氏は、このふたつの現象の因果関係の解明に取り組んだ。

申請者は、まず、アポトーシス性細胞容積減少時に開くことが知られている Cl^- チャネル VSOR のブロッカー存在下では、スタウロスポリン刺激による、細胞容積減少、MAPKK である ASK1 の下流にある p38 および JNK のリン酸化、細胞死に直結するカスペースの活性化、細胞死のいずれもが、抑制されることを観察した。さらに、スタウロスポリン刺激による細胞容積減少が、p38 のブロッカーによっても、RNAi 処理による ASK1 の発現低下によっても、抑制されないという極めて重要な知見を得た。これらの結果は、スタウロスポリン刺激による細胞死の誘導において、VSOR の活性化による細胞容積減少が、ASK1 活性化とそれに続く p38 および JNK のリン酸化よりも、上流にあることを明確に示したものである。

次のステップとして、申請者は、スタウロスポリン刺激によらない細胞容積減少時の現象の解析を行った。すなわち、HeLa 細胞を高浸透圧液に浸すと、直ちに、細胞内の水が細胞外に移動して細胞容積が減少するが、この状態からの回復をカチオンチャネルブロッカーにより抑制して、解析を行った。その結果、確かに、カスペースの活性化と細胞死がおこること、速い時間経過で p38 および JNK のリン酸化が高浸透圧の程度に依存して起こること、そして、p38 のリン酸化が、VSOR チャネルのブロッカーで抑制されないことを見いだした。これらの結果から、スタウロスポリン刺激による VSOR 活性化を介したアポトーシス性細胞容積減少時のみならず、高浸透圧刺激による VSOR 活性化を介さない持続的細胞容積減少時の場合にも、ASK1 活性化とそれに続く p38 および JNK のリン酸化がおこることが、明らかにされた。

本研究におけるこれらの知見は、細胞死の研究分野における未解決の重要問題に対して、確実なデータを提示して明確な解を与えたものであり、その意義は大きい。さらに、今後、本研究を土台に、細胞容積減少がどのようにして ASK1 を活性

化するのかという、細胞生物学的に興味深い問題の解決に展開していくことが期待できる。以上の理由から、審査委員会は、全員一致で、本論文が学位論文として相応しいものであると判断した。