PKN1 controls the aggregation, spheroid formation, and viability of mouse embryonic fibroblasts in suspension culture

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(Degree)
博士（医学）

(Date of Degree)
2020-03-25

(Resource Type)
doctoral thesis

(Report Number)
甲第7738号

(URL)
https://hdl.handle.net/20.500.14094/D1007738

(課程博士関係)

学 位 論 文 の 内 容 要 旨

PKN1 controls the aggregation, spheroid formation, and viability of mouse embryonic fibroblasts in suspension culture

PKN1 は、浮遊培養におけるマウス胚線維芽細胞の凝集、スフェロイド形成、および生存率を制御する。

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Introduction

Protein kinase N (PKN) is a serine/threonine protein kinase with a catalytic domain homologous to protein kinase C (PKC) and a regulatory region containing antiparallel coiled-coil (ACC) finger domains. PKN1, also known as PKNα or PRK1, is one of the three PKN isoforms (PKN1, PKN2, and PKN3) derived from different genes in mammals. PKN1 is an effector protein kinase of the Rho family GTPases, such as RhoA, RhoB, RhoC, and Rac1, in mammalian tissues. Rho family GTPases play an important role as regulators of cell-cell adhesion in a manner which varies substantially depending on cell type and cellular context. PKN1 is also involved in cell-cell adhesion among mammary epithelial cells and in lymphocyte adhesion to vascular endothelial cells, whereas direct interactions between PKN1 and Rho family GTPases were not described in these cases. In this study, we prepared mouse embryonic fibroblasts (MEFs) from PKN1[T778A] mice deficient in PKN1 kinase activity due to the introduction of a point mutation (T778A) in the enzyme's activation loop and analyzed the phenotype of MEFs compared with wild-type MEFs to elucidate the role of kinase activity of PKN1 in fibroblasts. We focused on cell aggregation/spheroid formation of MEFs in suspension culture which is one of the commonly used 3D culture methods. Aggregate/spheroid formation from fibroblasts in suspension culture is regarded as a good *in vitro* model for the fibroblasts in *in vivo* pathological conditions such as cancer and inflammation.

Results

PKN1[T778A] MEFs show delayed aggregate formation in suspension culture in flat-bottom poly-HEMA coated dishes

At first, morphological features of WT and PKN1[T778A] MEFs were investigated in attached and detached culture condition. Phase-contrast microscopy of cells in normal attachment culture conditions did not show significant difference between WT and PKN1[T778A] MEFs. Suspension culture of WT MEFs in flat-bottom poly-HEMA coated dishes time-dependently formed cell aggregates within a few hours. On the other hand, PKN1[T778A] MEFs showed significantly delayed aggregation in suspension culture. Additionally, PKN1[T778A] MEFs aggregates formed within 24 hours had smaller number and area compared with WT MEFs. To investigate the cause of delayed aggregation, we analyzed cell behavior by video imaging from 20 min after plating in flat-bottom poly-HEMA coated dishes. The average velocity and distance covered of each cell were significantly reduced in PKN1[T778A] MEFs than in WT MEFs. Cell-cell contact in PKN1[T778A] MEFs occurred less frequently compared with WT MEFs. Thus, we artificially increased the frequency of cell-cell contact by shaking the cell culture in a graded manner. Suspension cultures of PKN1[T778A] MEFs shaken at 60 revolutions per rpm still failed to form aggregates. By increasing the speed from 60 to 120 rpm, PKN1[T778A] MEFs came in close contact with each other and finally formed aggregates like WT MEFs at 180 rpm. Results suggest that less frequency

of cell-cell contact due to impaired cell motility contributes to the delayed aggregate formation of PKN1[T778A] MEFs in suspension culture.

PKN1[T778A] MEFs show susceptibility to anoikis in suspension culture

We examined whether inactivation of PKN1 in MEFs affect cell viability in suspension culture in flat-bottom poly-HEMA coated dishes. Cell viability was evaluated by quantifying the DNA content using a fluorescence-based method and the cellular ATP level using a luminescence-based method. Both DNA and ATP contents increased time-dependently in parallel for both WT and PKN1[T778A] MEFs under attachment culture condition, suggesting no difference in the cell viability between the two. However, both DNA and ATP contents decreased time-dependently in parallel for both WT and PKN1[T778A] MEFs in suspension culture in flat-bottom poly-HEMA coated dishes, suggesting that both underwent cell death. Particularly, PKN1[T778A] MEFs has significantly less DNA and ATP contents, suggesting that these were more susceptible to cell death than WT MEFs. These results imply that PKN1 activity contributes to resistance to anoikis. Previous reports stated that anoikis can be suppressed through cell-cell adhesion by a variety of cells. Therefore, the delayed cell aggregation observed in PKN1[T778A] MEFs may be causing the higher susceptibility to cell death in suspension culture.

PKN1[T778A] MEFs show impaired compaction for spheroid formation in suspension culture

To examine if PKN1 is involved in the compaction stage of MEFs, we used U-shaped ultra-low-attachment (Prime Surface) well plates that reportedly support growth and formation of tight spheroids or compact aggregates in a variety of cell lines. Initially larger-sized and loosely packed WT MEF aggregates underwent time-dependent compaction into a single round spheroid. However, PKN1[T778A] MEFs revealed irregular compaction with significantly less roundness, solidity, and sphericity than WT MEFs before 24 hours and only formed the compact spheroid like WT MEFs after 48 hours. This suggests that PKN1 is required for regular compaction process but not for the resultant compact spheroid of MEFs. We also examined if PKN1 is involved in cell viability during the compaction process in U-shaped ultra-low-attachment well plates. PKN1[T778A] MEFs has significantly decreased ATP content than WT MEFs, suggesting that PKN1[T778A] MEFs are more susceptible to cell death during compaction stage.

Surface expression of N-cadherin and integrins α5 and αV are downregulated in PKN1[T778A] MEFs

Cadherin is one of the most critical molecules responsible for the formation and maintenance of cell-cell contact. Therefore, we checked the possibility of involvement of cadherin in the observed difference of aggregation and compaction between WT and PKN1[T778A] MEFs. Both WT and PKN1[T778A] MEFs

expressed almost undetectable levels of E-cadherin by immunoblotting, corroborating the report stating that fibroblasts express N-cadherin, whereas E- and -P cadherins are undetectable. Thus, we compared the surface expression of N-cadherin on WT and PKN1[T778A] MEFs collected 30 min after starting suspension culture using flow cytometry. Surface N-cadherin expression level evaluated by mean fluorescent intensity (MFI) was significantly lower in PKN1[T778A] MEFs than WT MEFs.

Integrins α5, αV, and β1 are fibronectin-binding integrin subunits highly-expressed in fibroblasts. It was also reported that fibroblasts treated with anti-β1 and anti-α5 antibodies formed loose spheroids. Thus, we examined the surface expression of integrins α5, αV, and β1 on MEFs using flow cytometry. MFI of integrins α5 and αV were significantly lower in PKN1[T778A] MEFs than WT MEFs. These results taken together suggests that, the lower expression of N-cadherin and decreased surface expression of integrins might contribute to the abnormal aggregate/spheroid formation of PKN1[T778A] MEFs.

Conclusion

PKN1 has been paid attention as a promising therapeutic target of cancer, based on its role in migration of cancer cells. Our study suggests that PKN1 has important roles not only in cancer cells but in fibroblasts, essential components of cancer microenvironment. Further investigation regarding the effect of PKN1 inactivation on the activity of aggregate/spheroid of fibroblasts (e.g. production of cytokines, growth factors, and proteases) may help elucidate a new mode of action and the therapeutic or adverse effects of PKN1 inhibitors.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲第 2955 号	氏名	MEHRUBA
論文題目 Title of Dissertation	PKN1 controls the aggregation, spheroid formation, and viability of mouse embryonic fibroblasts in suspension culture PKN1 は浮遊培養におけるマウス胚線維芽細胞の凝集、スフェロイド形成、および生存率を制御する。		
審査委員 Examiner	主査 Chief Examiner 青井貴之 副査 Vice-examiner 横崎宏 副査 Vice-examiner 田守義和		

（要旨は１，０００字～２，０００字程度）

本研究で申請者らは、マウス胎仔線維芽細胞（MEF）の浮遊培養による細胞凝集およびスフェロイド形成における PKN1 のリン酸化酵素活性の機能について検討した。

申請者らは PKN1 の活性中心に点突然変異を有することによりリン酸化酵素活性を喪失したマウスの MEF（PKN1[T778A] MEF）を用いて研究を行った。通常の接着培養では、野生型 MEF と PKN1[T778A] MEF の間には形態的に明らかな違いはなかった。次に、poly-HEMA でコートすることにより接着性を低減した培養皿を用いた浮遊培養を行ったところ、野生型 MEF では数時間の間に細胞凝集塊を形成した。一方、PKN1[T778A] MEF を同様の条件で培養すると、24 時間以内に形成された細胞凝集の数と面積はいずれも野生型 MEF のそれらと比較して小さかった。このような差が生じる原因を明らかにするため、経時的撮影とその解析を行ったところ、PKN1[T778A] MEF では野生型 MEF と比べて、平均移動速度は遅く、平均移動距離が短いことが分かった。このことにより、PKN1[T778A] MEF の浮遊培養においては、野生型 MEF のそれと比べて、細胞 - 細胞間の接触が起こる頻度が少ないものと考えられた。

次に申請者らは、MEF による PKN1 の活性喪失は上述の培養条件において細胞の生存に影響を与えるか否かを調べた。細胞生存は DNA 量と細胞内 ATP 量を定量することで評価した。その結果、野生型 MEF と PKN1[T778A] MEF のいずれにおいても、時間経過にともなって DNA 量と ATP 量は減少したが、PKN1[T778A] MEF においてより大きな減少がみられた。このとから、PKN1[T778A] MEF では野生型 MEF と比べて細胞死が起こりやすいことが示唆された。

申請者らは次に、初期にみられる分散細胞の loose な凝集から tight なスフェロイド形成への移行に焦点をあてて検討を進めるため、低接着性でU字型の底面をもつ培養皿に MEF を播種した。播種後 24 時間の時点で野生型 MEF と比べて、PKN1[T778A] MEF では真円度や硬さ、真球度が低い不整なコンパクションがみられた。フローサイトメトリーによる解析により、PKN1[T778A] MEF では野生型 MEF と比べて細胞表面における N カドヘリンやインテグリンα5 およびαV の発現が低いことが分かり、これが細胞凝集やスフェロイド形成の不全の原因になっていると考えられた。

本研究は、PKN1 について、MEF の浮遊培養による細胞凝集およびスフェロイド形成におけるそのリン酸化酵素活性の機能について研究したものであるが、従来はほとんど行われなかった間葉系細胞の細胞 - 細胞間相互作用の分子機構における PKN1 の意義について重要な知見を得たものとして価値ある集積であると認める。よって、本研究者は、博士（医学）の学位を得る資格があると認める。