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学 位 論 文 名	Rapid transport of plasmid DNA into the nucleolus via actin depolymerization using the HVJ envelope vector. （HVJ-E はアクチンの阻害作用によってプラスミド DNA の核小体への輸送を促進する）
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論 文 内 容 の 要 旨

〔 目　　的 〕

Although nuclear transport of therapeutic genes is an essential requirement of human gene therapy, factors required for nuclear entry of DNA remain to be elucidated. Non-viral vector systems have led to numerous improvements in the efficiency of delivery of exogenous DNA into cells. However, nuclear transport of plasmid is difficult to achieve.

Hemagglutinating virus of Japan (HVJ ; Sendai virus) envelope vector was constructed by incorporating plasmid DNA into inactivated HVJ particles. This HVJ envelope vector (HVJ-E) introduced plasmid DNA efficiently and rapidly into the nucleus of various cell lines, although the nuclear transport mechanisms remain unclear.

Here, We examined nuclear translocation mechanisms of plasmid DNA delivered by HVJ-E to explain how HVJ-E transported plasmid DNA to the nucleus.

〔 方法ならびに成績 〕

First, we investigated localization of Cy3-labeled plasmid DNA (Cy3-pDNA) that was introduced into the cell by HVJ-E compare with Lipofectamine. At 120 min after transduction, The plasmid DNA transported by HVJ envelope vector located in the nucleus with characteristic particulate accumulation as well as in the cytoplasm while Lipofectamine-transduced plasmid DNA localized only in cytoplasm.

Then we performed a time-course analysis of localization of Cy3-labeled plasmid DNA (Cy3-pDNA) introduced directly into the cytoplasm of HeLa cells using HVJ-E. Statistical analysis indicated that 26% of the HVJ-E-transduced cells showed nuclear accumulation of the plasmid DNA at 20 min. after transduction, and this ratio rapidly increased to 87% in 120 min.

To investigate the specific focus of HVJ-E-introduced pDNA in the nucleus, we then examined the location of

Cy3-pDNA relative to the nucleoli. Immunofluorescent analysis using antibody against UBF, which is a nucleoli-specific protein complex, revealed co-localization of UBF with HVJ-E -mediated Cy3-pDNA in the nucleus.

Next, we examined whether gene expression were achieved from DNA introduced using a HVJ-E. In situ hybridization to detect luciferase mRNA was done at various intervals after HVJ-E containing pG13 luciferase gene transfection. The result showed the ratio of luciferase mRNA-positive cells was 4.9%, 51.4% and 82.4% at 2, 4 and 24 hr after transfection. The luciferase activity also was measured at various time points after HVJ-E G13 transfection. The data indicate that the plasmid DNA which mediated by HVJ-E in the nucleus, particularly the nucleolus, cause induction of gene expression.

Next, we compared the localization of Cy3-pDNA following microinjection alone, with that observed after microinjection and HVJ treatment. By microinjection alone, Cy3-pDNA was present only in the cytoplasm. But, following treatment with empty HVJ-E after microinjection, Cy3-pDNA fluorescence became apparent in the nucleus at 30 min, and much clearer at 120 min after injection. This fluorescence was confined to particulate structures which determined to be nucleoli.

Since recent reports have indicated that the actin cytoskeleton plays a significant role in limiting DNA mobility in the cytoplasm, we examined the assembly of actin filaments in HeLa cells before and after HVJ-E treatment. The depolymerization of actin filaments was clearly observed immediately after the HVJ-E treatment, and re-polymerization of actin was observed 30 min after the removal of HVJ-E in culture. On the other hand, HVJ-E treatment did not significantly alter the structure of Lamin B of the nuclear envelope of HeLa cells.

Similar depolymerization of actin is known to result from treatment with cytochalasin D (0.025 μ g/ml). We attempted to determine the particular conditions required for actin depo]ymerization within HeLa cells as a result of treatment with cytochalasin D, and determined that sufficient actin depolymerization was induced by 60 min of incubation with cytochalasin D at a concentration of 0.025 μ g/ml.

We then evaluated the effect of cytochalasin D on the nuclear entry of plasmid DNA introduced by microinjection. Cytochalasin D was added to the culture medium 30 min prior to microinjection of Cy3-pDNA into the cytoplasm of HeLa cells. With cytochalasin D treatment, Cy3-pDNA started to accumulate in the nucleus 60 min after microinjection, revealing a significant acceleration of nuclear entry, compared to the rate of nuclear entry observed after microinjection of Cy3-pDNA without cytochalasin D.

These results suggest that the actin cytoskeleton can inhibit the movement of pDNA in the cytoplasm, and that depolymerization of the actin cytoskeleton can enhance translocation of pDNA to the nucleolus.

〔 総　　括 〕

We demonstrated that plasmid DNA delivered by the HVJ-E vector rapidly reaches the nucleolus by inducing depolymerization of the actin cytoskeleton.

The HVJ-E vector delivers plasmid DNA by membrane fusion, and induces gene expression in cultured cells and tissue including non-dividing neuronal cells. Although this indicates that plasmid DNA is translocated into the nucleus but the mechanism remains unclear. Our present results suggest that the expression of plasmid DNA results from rapid transport of the DNA into the nucleolus by the HVJ-E vector.

HVJ induced transient actin depolymerization, Cytochalasin D, which induced actin filament depolymerization, also enhanced the transport of DNA into the nucleolus after microinjection. We concluded that depolymerization of the actin cytoskeleton induced the transport of plasmid DNA into the nucleolus.

Dauty et al found that the integrity of the actin cytoskeleton determined the mobility of DNA in the cytoplasm. This finding is consistent with the present results, however, they did not examine the nuclear transport of DNA

with regard to actin depolymerization. Therefore, a route to transport plasmid DNA into the nucleolus after actin depolymerization may exist. It is possible that this movement is specific for plasmid DNA lacking a NLS sequence. In an experiment in which DNA was conjugated with a NLS-containing protein, p50 of NFkB, followed by microinjection into the cytoplasm of HeLa cells, DNA reached the nucleus and was localized within the nucleoplasm, not the nucleolus. Thus, it is thought that some molecules, such as DNA, may be transported into the nucleolus in the absence of NLS when the actin cytoskeleton is depolymerized, although the mechanism by which this occurs is still unclear. However, DNA delivered by the HVJ-E vector moved into the nucleolus more effectively, compared with following microinjection, even when the cells were treated with cytochalasin D or empty HVJ-E vector after microinjection. Therefore, it is likely that the HVJ-E vector may have an additional enhancing effect with regard to movement of DNA into the nucleolus.

We thus found that HVJ can produce transient actin depolymerization in the initial stages of infection, however, the biological significance of actin depolymerization in the initial stages of infection remains unclear.

The HVJ-E vector can deliver genes directly to the cytoplasm by membrane fusion, which avoids degradation of exogenous genes. Another advantage is that the vector can achieve rapid transfer of DNA into the nucleus, thus overcoming a significant obstacle of using non-viral gene delivery vectors, thereby inducing efficient gene expression *in vivo*.

論文審査の結果の要旨

治療遺伝子の核内導入は遺伝子治療における重要な課題であるが、特に非ウイルスベクター法では十分克服されていない。本研究では、HVJ envelope vector（HVJ-E）によって導入された遺伝子の細胞内での動きを Cy3 標識プラスミド DNA を用いて解析した。標識 DNA は導入 120 分後に 80%以上の細胞の核小体に局在した。正電荷リポソームではこのような迅速な核内移行は見られなかった。導入されたルシフェラーゼ遺伝子の発現はルシフェラーゼ mRNA の in situ hybridization と酵素活性測定により確認された。マイクロインジェクションで細胞質に遺伝子を注入後、空の HVJ-E を作用させると核小体への移動が促進された。細胞骨格の遺伝子の動きに対する阻害作用、HVJ によるアクチン脱重合作用がすでに報告されている。そこでアクチン脱重合を起こす cytochalasin D を処理した細胞にマイクロインジェクションによりプラスミド DNA を注入すると、導入後 120 分で核小体への遺伝子の移動が認められた。以上より、HVJ-E による細胞質から核への遺伝子移動の促進はアクチン脱重合によることが明らかになった。

学位に値するものと認める。