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学位論文題目 A study of the intracellular delivery of functional nucleic acids by faint electric treatment

(微弱電流処理による機能性核酸の細胞内送達に関する研究)

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論文内容の要旨

Abstract

Effective delivery of macromolecular drugs especially functional nucleic acids into the target cells is important for the therapies of several diseases. However, some inherent properties of cells such as the lipophilic plasma membrane represent a dynamic barrier that restricts entry of extraneous hydrophilic and charged macromolecules into cells. In addition, inefficient endosomal escape is another obstacle to effective delivery of nucleic acids. Iontophoresis is a non-invasive transdermal drug delivery technology, which facilitates transdermal delivery of water soluble and ionized molecules by the application of faint electricity (0.5 mA/cm² or less) with electrodes on skin surface. The electric treatment on the skin provides driving force for transdermal delivery of drug molecules. Thus, the application of faint electric treatment (fET) would be a promising candidate for the intracellular delivery of nucleic acids. In this study, I examined the effects of fET on the cellular uptake and cytoplasmic delivery of functional nucleic acids.

I. Faint electric treatment-induced rapid and efficient delivery of nucleic acids into the cytoplasm

RNA interference (RNAi) is the basic endogenous mechanism for regulation of target gene expression in cells activated by small interfering RNA (siRNA). To explore whether fET induces cellular uptake of functional nucleic acids, the RNAi effect from fET of cells stably expressing luciferase in the presence of naked anti-luciferase siRNA was examined. Significant RNAi effect obtained from fET (0.34 mA/cm²) of cells indicates that fET can deliver naked siRNA into the cytoplasm of cells. Cytoplasmic delivery after fET was analyzed by confocal laser scanning microscopy (CLSM) using an oligonucleotide probe, in-stem molecular beacon (ISMB) that fluoresces upon binding to the complementary mRNA in cytoplasm. Interestingly, ISMB fluorescence appeared rapidly and homogeneously in the cytoplasm of cells when fET of cells expressing luciferase was performed with naked ISMB-luciferase. This result indicates that fET markedly enhances cytoplasmic delivery. In addition, the comparative study of Lipofectamine 2000 (LFN) based delivery of ISMB and fET based delivery of ISMB

showed that significant fluorescent intensity was observed in cells with fET even at 15 min and over 4-fold higher fluorescence than that of LFN at 30 min. Moreover, fluorescent signals were observed in all cells treated with fET/ISMB, whereas heterogeneous distribution of fluorescent signals was recognized in the cells after transfection of ISMB/LFN lipoplexes. These results indicate that fET delivered ISMB homogeneously into cytoplasm with faster speed than LFN. Cytotoxicity of fET method was evaluated by trypan blue exclusion test. Cells immediate after fET were not stained by trypan blue dye, indicating that fET did not induce electricity based cytotoxicity such as electroporation, membrane damage, and cell death. Mechanistic study demonstrated that macropinocytosis inhibitor, amiloride, caveolae-mediated endocytosis inhibitor, filipin, and low temperature exposure significantly suppressed fET-mediated cellular uptake of fluorescent-labeled siRNA, indicating that cellular uptake mechanism mediated by fET is endocytosis. In addition, voltage sensitive dye DiBAC4 (3) uptake was increased by fET, suggesting that fET would alter the membrane potential. Moreover, non-specific cationic ion channel such as transient receptor potential channel (TRP) inhibitor SKF96365 reduced uptake of fET-induced fluorescent-labeled siRNA. These results indicate that TRP channels would be involved in fET based cellular uptake process, in which the alteration of membrane potential occurs via the activation of TRP channel, leading to the cellular uptake of nucleic acids.

II. The novel functional nucleic acid iRed effectively regulates target genes following cytoplasmic delivery by faint electric treatment

Intelligent shRNA expression device (iRed) is a newly synthesized nucleic acid. It contains the minimum essential components needed for shRNA expression in cells such as U6 promoter and shRNA-encoding region, in which any one type of adenine (A), guanine (G), cytosine (C), or thymine (T) nucleotide unit was substituted by each cognate 4'-thio derivatives. iRed is expected as a novel tool for the regulation of target genes. However, general delivery carriers consisting of cationic polymers/lipids could impede function of a newly generated shRNA via electrostatic interaction in the cytoplasm. To overcome difficulties of iRed delivery, I examined the effect of fET on the cells stably expressing luciferase in the presence of iRed encoding anti-luciferase shRNA. Transfection of lipofectamine 2000 (LFN)/iRed lipoplexes showed an RNAi effect, while fET-mediated iRed transfection did not suppress luciferase activity of cells. The difference between siRNA/ISMB and iRed was attributed to their molecular sizes. The observation of intracellular trafficking of different-sized molecules such as FITC-dextran 10,000 and FITC-dextran 70,000 after fET suggested that endosomal escape efficiency of materials internalized by fET depends on the molecular size. Thus, significantly larger molecular size of iRed than siRNA and ISMB is likely to prevent endosomal escape after fET-mediated endocytosis. To improve endosomal escape of iRed taken up by endocytosis, fET with iRed was performed in the presence of chloroquine, which is known as lysosomotropic agent that accumulates in endosomes and lysosomes and enhances cytoplasmic delivery of various compounds. Interestingly, fET in the presence of chloroquine significantly increased the RNAi effect of iRed/fET to levels that were higher than those for the LFN/iRed lipoplexes. Furthermore, the amount of lipid droplets in adipocytes significantly decreased after fET in the presence of chloroquine with iRed against resistin that is a key adipokine of adipocyte maturation. The confocal microscopy of the intracellular trafficking of FITC-labeled iRed showed that green dots (FITC-labeled iRed) co-localized with red dots (endosomes/lysosomes) in the cells after fET without chloroquine, while the green fluorescence was widely distributed in the cells after fET with chloroquine. This indicates that endosomal escape of iRed was significantly increased after fET with chloroquine. Thus, fET of iRed carrying shRNA against resistin in the presence of chloroquine can suppress lipid accumulation during adipocyte maturation, suggesting that a combination of iRed with fET is a useful method for

effective regulation of target gene expression.

Conclusion

From these findings, fET can enhance cellular uptake and rapid cytosolic delivery by inducing energy-dependent pathways along with the activation of cationic channels. Thus, faint electric treatment could be useful for effective and safe delivery of functional nucleic acids into the cytoplasm of target cells.

審査の結果の要旨

イオントフォレシスは、微弱電流の印加によって荷電性物質を非侵襲的に皮内に送達する技術である。この微弱電流処理 (faint electric treatment, fET) による経皮送達は、投与コントロールが可能ならぬコンプライアンスが良好であるなどの多くの利点を有しており、従来適用が困難であった機能性高分子医薬品への展開が期待されている。本研究では、機能性核酸の新規細胞内送達技術として微弱電流処理の有用性を検討するとともに、その分子メカニズムの解析を行った。

1. 微弱電流処理による機能性核酸の細胞内送達機構

ルシフェラーゼ遺伝子発現細胞を用いて、微弱電流による small interfering RNA (siRNA) の細胞取り込みを詳細に検討した。種々の細胞取り込み機構阻害剤を用い、蛍光標識化 siRNA の微弱電流による細胞取り込み機構を検討した結果、微弱電流による核酸取り込みにはエンドサイトーシス等が関与していることが示唆された。また、細胞質中で標的 mRNA と結合すると蛍光を発する in-stem molecular beacon を用いて細胞質送達を評価したところ、微弱電流処理後直ちに外来機能性核酸が細胞質に到達することが確認された。さらに、機能性核酸の細胞取り込み活性化機構を検討したところ、微弱電流処理によって細胞膜電位が変化しており、また transient receptor potential (TRP) イオンチャネルタンパク質が関与する可能性を新たに見出した。

2. 微弱電流処理による新規機能性核酸 iRed の細胞内送達と機能発現制御

次に、微弱電流による新規機能性核酸 (intelligent shRNA expression device, iRed) の細胞内送達を解析した。抗ルシフェラーゼ shRNA を発現する iRed 存在下でルシフェラーゼ発現細胞を微弱電流処理したところ、有意な RNA 干渉効果はみられなかった。この原因として、iRed は siRNA よりも分子量が大きいためエンドソーム脱出効率が低いことが推察されたため、エンドソーム脱出促進剤であるクロロキン存在下で微弱電流処理を行ったところ、市販の lipofectamine 2000 (LFN)/iRed 複合体よりも高い RNA 干渉効果が認められた。さらに、脂肪細胞の成熟化に対する抗レジスチン iRed の細胞内導入を検討したところ、微弱電流処理とクロロキンの併用によって著しい脂肪滴蓄積抑制効果がみられ、疾患モデル細胞での有用性が確認された。

以上申請者は、微弱電流処理を利用した機能性核酸の効率的細胞内送達方法を見出すと共に、その分子メカニズムについての知見を得た。本知見は、機能性核酸の細胞内導入・機能発現制御技術の発展に大きく貢献する成果である。

学位論文とその基礎となる報文の内容を審査した結果、本論文は博士 (薬科学) の学位論文としての価値を有するものと判断する。