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臨床研究基盤整備推進研究事業

心筋微小血管造影装置の開発による糖尿病性心筋微小循環障害の可視化

（若手医師・協力者活用に要する研究）

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微小血管の可視化を目的とし、病院設置型微小血管造影装置の開発とその臨床応用を行った。ファントムを用いた検討では、理論上直径 50μm までの血管を描出することが可能であり、被検者の被曝量も、臨床上許容範囲にあることが判明した。血管新生療法の施行患者を対象として、治療前後における微小側副血管網の変化を比較検討した。

A. 研究目的

従来型の血管造影装置では描出できないような微小血管を観察することによって、様々な疾患の病態把握が可能となり得る。例えば、難知性の重症末梢動脈閉塞症に対する血管新生療法の臨床応用が行われているが、従来型血管造影検査では、新生血管の描出は不可能である。また、糖尿病性微小血管障害の病態把握や治療効果の判定に有効な検査はない。

本研究の目的は、病院設置型微小血管造影装置を開発、臨床応用することによって、微小循環障害の病態把握法や血管新生の新しい評価法を確立することである。

B. 研究方法

新エネルギー産業技術総合開発機構(NEDO)の支援のもと、浜松ホトニクス(株)を中心に、NHK エンジニアリングサービス、国立循環器病センター研究所、東海大学医学部等が協力して、病院設置型の微小血管造影装置を開発した。装置は、高出力の CT 用 X 線源とハイビジョンの高感度撮像系により構成されている。チャートを用いて、解像度を測定し、犬冠動脈のファントムで中核枝の評価およびウサギの虚血肢モデルでの再生血管の評価を行った。また、吸収線量および散乱線の測定を行い、安全性の検討を行った。臨床応用では、末梢動脈閉塞症に対する血管新生療法前後に微小血管造影を施行し、虚血下肢の微小血管を評価した。

(倫理面への配慮)

倫理委員会の審議・承認を得、本検査の合併症・効能・不利益・利益を説明し、本人及び家族の同意の元に施行した。

C. 研究結果

解像度の検討では、チャートにおいて、一般の血管造影では 250μm が限界であったが、病院設置型微小血管造影装置では、50μm まで観察できた。犬冠動脈のファントムでは一般の血管造影では、第 3 分岐までしか描出できなかったが、病院設置型微小血管造影装置では、第 4 分岐以下まで明瞭に描出できた。血管新生療法を施行したウサギ虚血肢モデルでは、100μm 以下の蛇行した側副血管が描出でき、アデノシンに対する反応性も評価可能であった。安全性の検討では、1m の位置で 20 秒照射時の吸収線量は 600mSv で、散乱線も被写体から 1m の位置で 0.2mSv であった。これは、従来の血管造影装置と同等のレベルであり、臨床応用上、許容範囲にあるものと考えられた。ヒトに対する臨床応用として、血管新生療法を行う下肢末梢動脈閉塞症の患者を対象に、合計 8 回の微小血管造影を施行した。造影に伴う被曝線量は通常の血管造影と同レベルであることが判明した。微小血管造影によって通常の DSA 造影では描出困難な 100μm 以下の微小血管が鮮明に描出された。

DSA に比較して少なくとも 1−2 分枝末梢側の血管が描出可能であった。1 ヶ月から 1 年の間隔を置いて施行したフォローアップ造影における微小血管の再現性は良好であった。血管新生療法前後で微小血管網の発達を検討すると、治療後に明らかな血管新生が認められたのは 3 割の症例に過ぎなかった。

D. 考察

病院設置型微小血管造影装置の 1 号機は、通常の血管造影と同等の安全性を有している。また、その血管描出能は通常装置に比し優れていた。造影を繰り返し施行し得た症例における微小血管の再現性は良好であった。血管新生療法前後において必ずしも微小血管数の増加が認められないことから、本療法の作用機序における血管新生以外の因子の関与が示唆された。

E. 結論

本研究で開発された病院設置型微小循環造影装置によって 50μm レベルの微小血管が観察可能であり、その安全性や再現性に問題はなかった。微小循環障害を伴う疾患の評価や、末梢動脈閉塞症に対する血管新生療法の作用機序の解明、治療効果判定に有用な検査法と思われた。

F. 健康危険情報

特になし。

G. 研究発表

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研究成果の刊行に関する一覧表

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Potentiation of Regenerative Therapy by Non-Viral Vector, Gelatin Hydrogel

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Summary. Both gene therapy and cell transplantation are promising approaches for therapeutic angiogenesis. However, gene therapy must overcome biohazard of viral vectors, transfection efficiency, and premature tissue-targeting. Conventional cell therapy is insufficient in some cases because of small cell numbers, poor survival, impaired differentiation, etc. Endothelial progenitor cells (EPCs) play an important role in modulating angiogenesis and vasculogenesis. Here, we present a new concept for hybrid cell-gene therapy using a nonviral vector, gelatin. Genetically-modified EPCs may serve, not only as a tissue-engineering tool to reconstruct the vasculature, but also as a vehicle for gene delivery to injured endothelium. Thus, hybrid cell-gene therapy may be a new therapeutic strategy for the treatment of intractable cardiovascular diseases.

Key words. Cell therapy, Transplantation, Angiogenesis, Adrenomedullin, Gene therapy

Therapeutic angiogenesis is a promising strategy for the treatment of intractable cardiovascular diseases such as ischemic heart disease, peripheral vascular disease, and pulmonary arterial hypertension. Although gene therapy has been shown to be an effective approach for angiogenesis, it is still unsatisfactory because of biohazard of viral vectors, transfection efficiency, and premature tissue-targeting (St George JA 2003). Therefore, highly efficient and safe gene transfer is desirable. Recently, we developed a novel nonviral vector, gelatin which allows highly efficient and long-lasting gene transfer. Because positively charged biodegradable gelatin hold negatively charged plasmid DNA in its positively charged lattice structure, (Fukunaka Y 2002, Nagaya N 2003) DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer (Tokunaga N 2004).

Recently, transplantation of stem cells or progenitor cells has been shown to regenerate a variety of tissues. Endothelial progenitor cells (EPCs) have been discovered in adult peripheral blood (Asahara T, et al. 1997). EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, then migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ(Kawamoto A, et al. 2001). Transplantation of EPC induces therapeutic angiogenesis in ischemic heart or limb (Kawamoto A 2001. Murohara T, et al. 2000). However, some patients are refractory to conventional cell therapy because of insufficient cell number, poor survival, impaired differentiation, etc. Thus, a novel therapeutic strategy to enhance the angiogenic property of EPCs is desirable. Here, we present a new concept for hybrid cell-gene therapy using a nonviral vector, gelatin. Gene-modified EPCs may serve not only as a tissue-engineering tool to reconstruct the vasculature, but also as a vehicle for gene delivery to injured endothelium.

This chapter focuses on gelatin-mediated in vivo and in vitro gene transfer, and the rationale and preliminary results of combining cell (EPCs) and gene therapy for regenerative medicine.

Nonviral vector, gelatin

Tabata Y, et al. discovered biodegradable gelatin which controls the release of growth factors such as basic fibroblast growth factor. Positively charged biodegradable gelatin ionically link with negatively charged protein (Tabata Y 1999, Tabata Y 1987). Thus, the gelatin has been widely used as a carrier of proteins because of its capacity to delay protein degradation. Plasmid DNA is known to be negatively charged. Thus, we used the gelatin as a vector for gene therapy. Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of approximately 30μm, water content of 95% and an isoelectric point (pI) of 9 after swelling in water (Tabata Y 1999, Tabata Y 1987). After a 2-hour incubation period, positively charged gelatin held negatively charged plasmid DNA in its positively charged lattice structure (Fig.1**a** and **b**). DNA particles are released from the gelatin through its degradation.

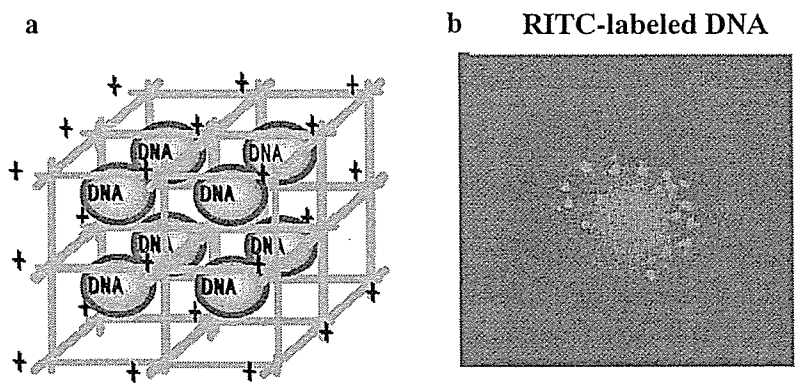

Fig. 1a, b. a Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. **b** A number of RITC-labeled AM DNA particles were incorporated into gelatin.

Angiogenic potential of adrenomedullin

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (Kitamura K 1993). AM and its receptor are expressed mainly in vascular endothelial cells and vascular smooth muscle cells. AM not only induces vasorelaxation but also regulates growth and death of these vascular cells (Nagaya N, et al. 2000. Nishimasu H, et al. 2001). A recent study has shown that vascular abnormalities are present in homozygous AM knockout mice, suggesting that AM is indispensable for vascular morphogenesis (Shindo T, et al. 2001). Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, is a multistep process that involves migration and proliferation of endothelial cells, remodeling of the extracellular matrix and functional maturation of the newly assembled vessels. Recently, AM induced tyrosine phosphorylation of Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase1/2 (ERK1/2) in human umbilical vein endothelial cells (Kim W, et al. 2003). Both signals play important roles in regulation of multiple critical steps in angiogenesis and vasculogenesis; endothelial cell survival, proliferation, migration, and capillary-like structure formation. These findings raise the possibility that AM gene transfer plays a role in modulating vasculogenesis and angiogenesis in ischemic tissues.

In vivo transfection

We examined the usefulness of gelatin as a nonviral vector for in vivo gene transfer (Tokunaga N, et al. 2004). AM plasmid DNA was used for therapeutic angiogenesis. We demonstrated that AM DNA was incorporated into positively charged gelatin. Interestingly, AM immunoreactivity surrounding AM DNA-gelatin complexes in the skeletal muscles was intense (Fig.2**a**). AM production of AM-gelatin group was enhanced compared with that of naked AM DNA group (Fig.2**b**). Furthermore, gelatin allowed long-lasting AM expression after gene transfer. These results suggest that biodegradable gelatin may serve as a nonviral vector for gene transfer. In fact, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hindlimb ischemia than naked AM DNA

(Fig.3), as evidenced by significant increases in histological capillary density, calf blood pressure ratio, and laser Doppler flow. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. AM DNA-gelatin complexes were distributed mainly in connective tissues. We have recently demonstrated that gelatin-DNA complexes are readily phagocytosed by macrophages, monocytes, endothelial progenitor cells etc, resulting in gene expression within these phagocytes (Tabata Y and Ikeda Y 1987). These findings raise the possibility that AM secreted from these cells acts on muscles in a paracrine fashion. Unlike AM production in the Naked AM group, AM overexpression in the AM-gelatin group lasted for longer than two weeks. Thus, it is interesting to speculate that delaying gene degradation by gelatin may be responsible for the highly efficient gene transfer. These results suggest that the use of gelatin, which is considered to be less biohazardous than viral vectors, enhances the angiogenic potential of AM DNA. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular diseases.

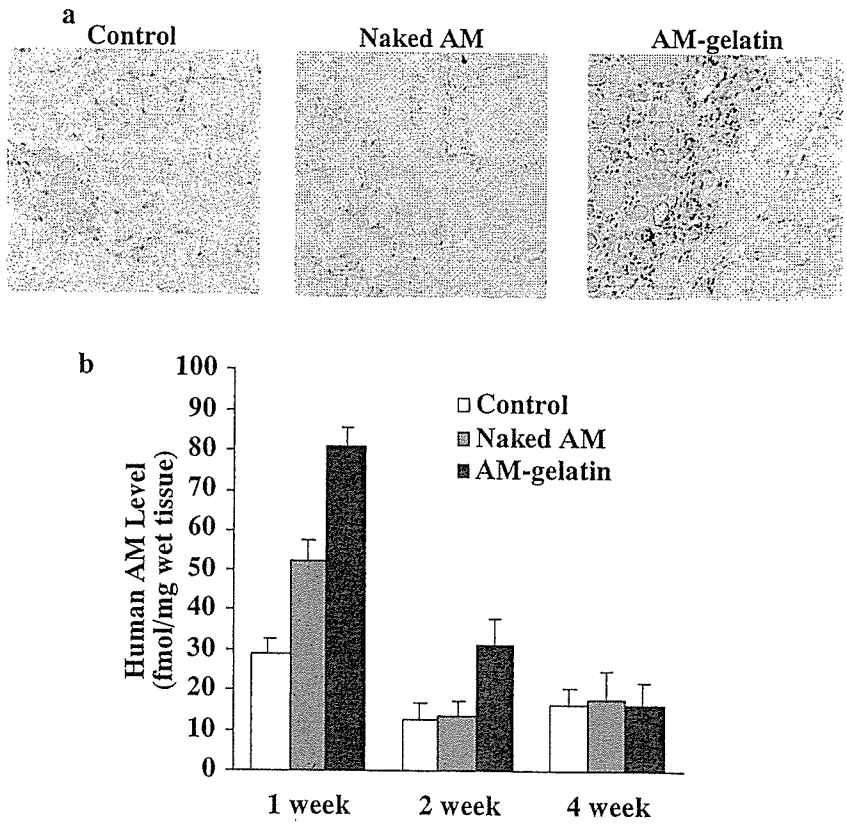

Fig. 2a, b. a Immunohistochemistry for AM 7 days after gene transfer. Intense immunostaining was observed surrounding gelatin in the AM-gelatin group. Magnification x200. **b** Time course of AM production in ischemic muscles after gene transfer. Data are mean ± SEM. *P < 0.01 vs Control group; †P < 0.01 vs Naked AM group.