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A Novel Micro-Angiography Detecting Angiogenesis, Application for Autologous Bone Marrow Mononuclear Cells Transplantation in the Patients with Critical Limb Ischemia

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Summary. Conventional Anigiographic Findings in Autologous Bone Marrow Mononuclear Cells Transplantation for Critical Limb Ischemia: Bone marrow mononuclear cells have many of the characteristics of stem cells for mesenchymal tissues, and secrete many angiogenic cytokines. We performed autologous transplantation of bone marrow mononuclear cells in six patients with critical limb ischemia due to Buerger disease, who were not candidates for catheter or surgical revascularization. Leg pains at rest and skin ulcers improved after bone marrow transplantation in all patients, although significant collateral developments after the therapy by conventional angiography could not be observed. Autologous transplantation of bone marrow mononuclear cells including stem cells improved critical limb ischemia due to Buerger disease. Neovascularization after therapeutic angiogenesis might be quite small and could not be visualized by conventional angiography.

Novel Micro-angiograpy:We developed in-hospital micro-angiographic equipment which consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera). We visualized mid-zone collaterals after femoral arterial exfoliation with and without therapeutic angiogenesis in rabbit ischemic limbs and assessed the radio-absorptions in a clinical setting. The micro-angiography clearly demonstrated mid-zone collaterals after the treatment with a diameter of down to 50μm, but the conventional angiography did not. The sum of ra-

dio-absorptions for 10 seconds in clinical settings was 300 mSv. The newly developed in-house micro-angiography could illuminate micro-vessels with a diameter of down to 50μm in clinical settings safely and could be useful in the evaluation of therapeutic angiogenesis.

Keywords. Micro-angiography, Angiogenesis, Autologous bone marrow mononuclear cells transplantation, Critical limb ischemia, Buerger disease

Introduction

Endothelial progenitor cells (EPCs) possess the ability to mature into cells that line the lumen of blood vessels(Asahara T, et al. 1997). Therapeutic angiogenesis could be induced by the transplantation of bone marrow mononuclear cells including EPCs. Several studies demonstrated that therapeutic angiogenesis using autologous bone marrow mononuclear cells transplantation (BMT) was effective for ischemic vascular diseases although conventional angiography could not precisely detect developed collaterals after therapeutic angiogenesis(Iba O, et al. 2002, Inaba S, et al. 2002, Shintani S, et al. 2001,Tateishi-Yuyama E, et al. 2002). We developed an in-hospital micro-angiographic equipment which consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera).

The purpose of the present study was to evaluate the clinical effects and conventional angiographic findings on BMT for critical limb ischemia, and to validate the usefulness and safety of the novel micro-angiography technique for the evaluation of therapeutic angiogenesis.

Methods

Patients

Patients qualified for autologous BMT if they had chronic critical limb ischemia including rest pain and/or non-healing ischemic ulcers for a minimum of 4 weeks without evidence of improvement in response to

conventional therapies and were not optimal candidates for surgical or catheter revascularization. Buerger's disease was diagnosed by segmental occlusion of small- and medium-sized arteries, absence of atherosclerosis, and corkscrew collaterals circumventing the occlusion in angiogram and the exclusion of autoimmune diseases such as scleroderma or systemic lupus erythematosus, hypercoagulable states, diabetes, or acute arterial occlusion secondary to embolism. Patients with retinopathy and/or malignancy were excluded. Although 30 patients with atherosclerotic peripheral artery disease were candidates for BMT, they were excluded from the present study due to their systemic atherosclerotic complications. Six patients with Buerger's disease were recruited for the present study. All patients had legs pain at rest and five patients had foot ulcers. Written consent was obtained from all participants of this study. This clinical trial of autologous BMT for the treatment of patients with critical ischemia was approved by the Medical Ethics Committee of the National Cardiovascular Center.

Autologous BMT

Bone marrow fluid (700-800ml) was collected from the iliac bone under general anesthesia. The harvested bone marrow fluid was diluted with RPMI 1640 (Nikken Bio Medical Laboratory, Kyoto, Japan) containing heparin, then stored in a sterile pack from the Bone Marrow Collection Kit (Baxter, IL, USA). The mononuclear cell fraction was prepared with a Fresenius AS104 (AMCO, USA). The injection volume was 0.5ml and injections were spaced 2-3cm apart, using a 1ml syringe and a 27-gauge needle. Leg pains were measured by a visual analog pain scale and foot ulcers were evaluated by area and appearance.

Novel micro-angiography

The in-hospital micro-angiographic equipment consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera) (Fig.1).

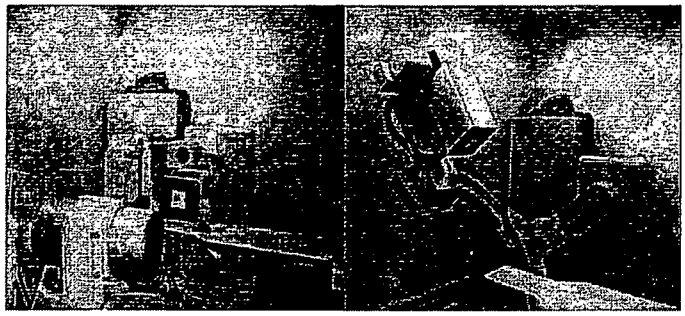

Fig. 1a, b. The micro-angiographic equipment that we developed. High-voltage power X-ray source **a** and a detecting system with a high spatial resolution(25μm) and high sensitivity (100 times of CCD camera) **b**.

Limb ischemia models in rabbits were made by ligating the femoral artery and treated by fibroblast growth factor 4 (FGF-4) genes incorporated to gelatin hydro gel (GHG). One month after the treatment, we evaluated collateral micro-vessels by using conventional and micro-angiographic systems. The approach was via the left femoral artery so that the catheter was located in the abdominal aorta. A 5ml bolus of Iodine contrast medium (300mg/ml) was injected at 3ml/sec using an auto-injection system. Imaging was recorded using a digital source in 1000 x 1000 pixels. The sum of radio-absorptions for 10 seconds in clinical settings was studied.

Results

Autologous BMT for Critical Limb Ischemia

The number of transplanted bone marrow mononuclear cells were one to five multiplied 10^9. Rest pains decreased or disappeared in one month after BMT (Fig.2) and Skin ulcers improved in one to three months after BMT in all patients (Fig.3).

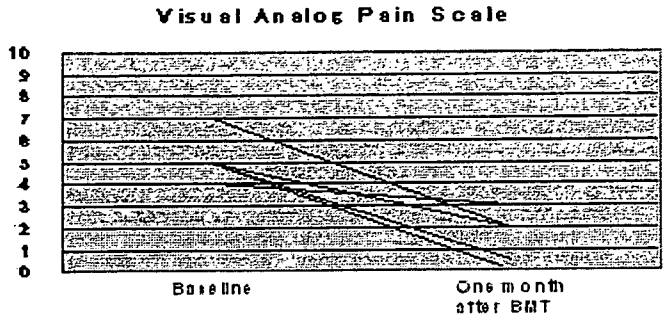

Fig. 2. The Visual analog pain scale in all patients.

Fig. 3a, b. The skin ulcers in a patient before **a** and one month after autologous bone marrow transplantation **b**.

Conventional angiography was performed before and one month after BMT, but there was no significant changes in any of the patients (Fig.4).

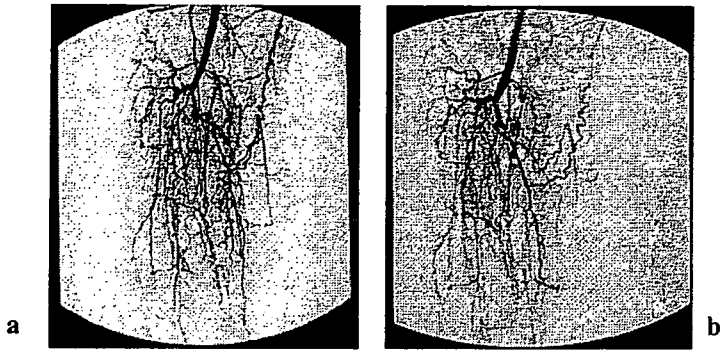

Fig. 4a, b. The conventional angiographic findings in the patient before **a** and one month after autologous bone marrow transplantation **b**.

Novel micro-angiography

The novel micro-angiography can detect to within a limit 50 of μm, although a detection limit of a conventional angiography is 250μm (Fig.5).

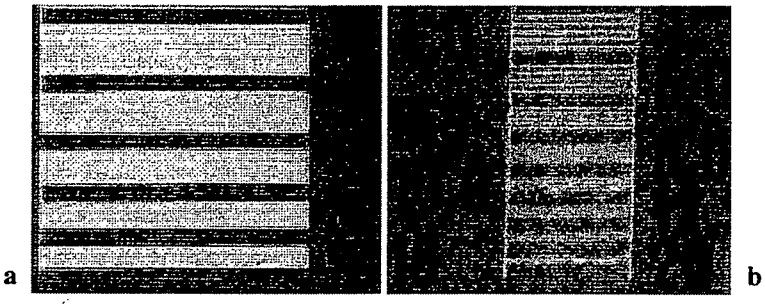

Fig. 5a, b. The detection limits on a conventional angiography **a** and the novel micro-angiography **b** using a line chart

Collateral micro-vessels, which were 100-500μm or less in diameter, were demonstrated more clearly in micro-angiography than conventional angiography (Fig.6).

The sum of radio-absorptions at the point of 1m distance from the X-ray source in clinical settings was 300 mSv. for 10 seconds.

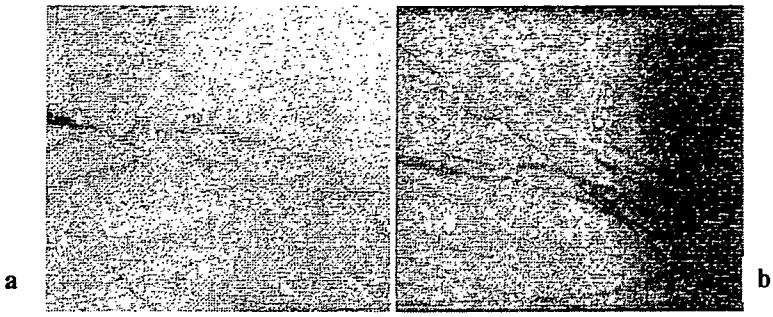

Fig. 6a, b. In 2.5x2.5cm view size, Collateral micro-vessels after therapeutic angiogenesis in the rabbit limb ischemia model. Vessel sizes in the range of 100-500μm or less, were demonstrated in the novel micro-angiography b more clearly than in a conventional angiography a. The diameter of the line in the micro-angiography is 130μm.

Discussion

Autologous BMT improved chronic severe limb ischemia due to Bue rger's disease. Conventional angiography could not disclose develope d collateral vessels after BMT. A novel micro-angiography technique could illuminate promoted collateral vessels after therapeutic angioge nesis in rabbit models although a conventional angiography did not. The sum of radio-absorptions in the novel angiography could be acc-epted in clinical settings.

Autologous BMT and Buerger's disease

Bone marrow harvests need an amount of more than 500ml bone marrow fluid and general anesthesia in therapeutic angiogenesis using BMT. Such factors have practical limitations to select candidates with peripheral artery disease complicated with systemic atherosclerosis and aging for BMT. Buerger's disease is a segmental vasculitis that affects the distal arteries of the upper and lower extremities. It typically occurs in young people. The majority of patients with Buerger's disease have pain at rest and digital

ulcerations and are hard to treat by revasculaizations, including catheter angioplasty and surgical bypass grafting, because of peripheral artery lesions. Patients with Buerger's disease, however, tend to have less systemic atherosclerotic lesions and normal cardiac function. These suggest that patients with Buerger's disease are the ideal candidates for therapeutic angiogenesis using autologous BMT.

Discrepancy between clinical improvements and conventional angiographic findings after BMT

BMT improved critical limb ischemia clinically. Promoted collateral vessels after the treatment were not, however, visualized well by conventional angiography. These vessels are quite small and the detection limit of small vessels by conventional angiography is about 200μm in diameter.

Novel micro-angiography

Recently, synchrotron radiation system characterized by high brightness, monochromatic and collimated nature bypass, revealed micro-vessels in situ. However the high cost of a synchrotron system strictly limits its clinical application (100 million dollars or more). We developed an in-house micro-angiographic system with a relatively low cost of approximately 1million dollars, which consisted of a high-voltage power X-ray source and a detecting system with a high spatial resolution (25μm) and high sensitivity (100 times of CCD camera). We evaluated collateral micro-vessels one month after therapeutic angiogenesis by using the conventional and micro-angiographic system. The in-house micro-vessel angiographic system could detect the micro-vessels more precisely than conventional angiographic system. We thought that the present micro-angiography should be useful for evaluating efficacy of therapeutic angiogenesis in clinical settings.

Conclusions

Conventional angiography failed to disclose the promoted collateral vessels after BMT although BMT improved the critical limb ischemia clini-

cally. The in-house micro-angiographic system could detect the micro-vessels more precisely than conventional angiographic system and the sum of the radio-absorption in the equipment could be acceptable in clinical settings. The novel in-house micro-angiographic system can be useful in the evaluation of therapeutic angiogenesis clinically.

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4．画像解析-微小血管造影-

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狭心症や心筋梗塞などの虚血性心疾患や閉塞性動脈硬化症に対する新しい治療戦略として血管再生治療[用解1)]が期待されている。実際の臨床では，血管造影を含めた臨床検査で臨床症状の効果を十分には反映していない。これは，既存の血管造影装置の解像度は約200～300 μmであり，再生される新生血管は約100 μm以下の微小血管であるからである。微小血管造影の先駆けとなったシンクロトロンによる微小血管造影法[用解2)]は200～500 μm以下の微小血管の定量と50～200 μm以下の微小血管の可視化が可能である。さらに，臨床の場で簡便に使用できる微小血管造影装置も開発された。本稿では，再生治療後の微小血管の評価方法について概説する。

はじめに

血管再生には，一般に既存の血管から血管内皮細胞が増殖，リモデリングし，新しい血管枝が形成される狭義の血管新生（angiogenesis）と，血管内皮前駆細胞である血管芽細胞が集合・分化して血管が形成される血管発生（vasculogenesis）の2つが考えられている。血管発生は，主に胎生期に行われると考えられていたが，成人末梢血中のCD34陽性単核球の分画から血管内皮細胞へと分化しうる血管内皮前駆細胞（endothelial progenitor cell：EPC）があることが報告され，成人においても血管発生による血管再生が起こりうることが示された[1)2)]。特に単核球分画中で血管内皮細胞に分化しうる単核球は，主に骨髄に存在するため，動物実験の虚血モデルに骨髄単核球細胞移植をすることにより，血管新生や側副血行路が発達し，下肢血流量増加作用や心機能が改善することが確認された。これに基づき，重症下肢虚血患者に対し自己骨髄細胞移植や末梢血幹細胞移植が臨床導入され，良好な成績が報告されている[3)]。しかし，既存の血管造影装置では空間解像度が200 μm前後で，ミリメーターオーダーの血管を主たる観察対象としている。そのため，新生血管床の構築と機能の評価には極めて不十分といわざるを得ない。そこで，微小血管を観察できる微小血管造影法に期待がもたれている。

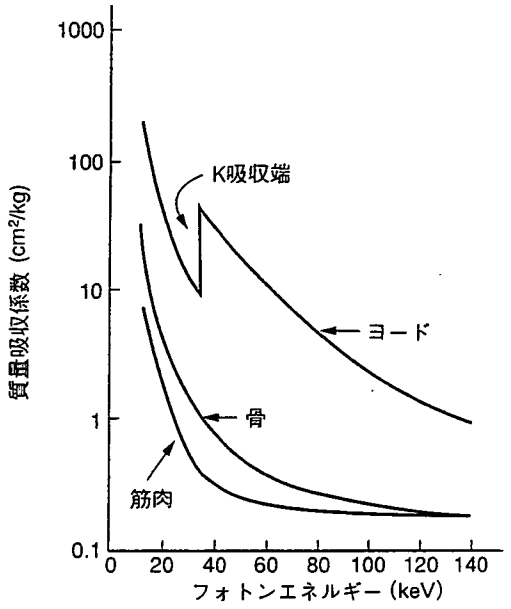
Ⅰ．微小血管造影法

1．放射光微小血管造影法

再生された微小血管を血管造影検査で評価するためには，微量の造影剤を検出できる装置が必要となる。その要素としてはX線の性質が高輝度で，平行性，単色性であり，なおかつ検出系が高感度，高解像度であることが重要である。これらの要素をすべて取り揃えているのが放射光施設内の微小血管造影装置である[4)]。放射光とは広域のスペクトルを持つ白色光であり，太陽光のように限りなく

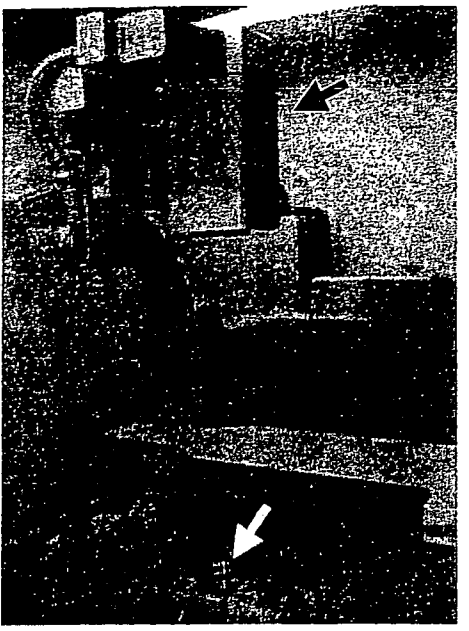
key words
微小血管造影，血管再生治療，新生血管，単色X線，放射光微小血管造影法，病院設置型微小血管造影法，プラズマX線微小血管造影法，angiogenesis，vasculogenesis，endothelial progenitor cell

図❶　X線エネルギーと質量吸収係数



筋肉，骨およびヨードのK吸収端の関係。ヨードのK吸収端は33.3keVで上昇するため，その直上で人体との組織吸収係数の差が最大となる。

図❷　病院設置型微小血管造影装置



NHKの高感度・高精細撮像管であるアバランシェ型ハイビジョンモノクロ新Super-HARPカメラ（黒矢印）とCT用のX線管を転用した高出力線源（白矢印）から構成されている。

平行に近い性質がある。単色光の利点として，ヨードは33.3keVのエネルギーレベルでK吸収端を持つ。これは質量吸収係数が不連続に上昇し，X線のエネルギーをヨードのK吸収端の直上のエネルギーに変換すると，ヨードと周囲組織との質量吸収係数の差が最大となる（図❶）。組織とヨードとのコントラストが最良となるため，微量のヨードを検出できやすくする効果がある。放射光のX線は，既存のX線装置より約108倍以上も輝度が高く，シリコン結晶を用いてヨード吸収端の直上に設定することにより，単色化しても十分なフォトン量を維持することが可能である。検出系は高解像度・高感度蛍光板で作製した蛍光像を，超高感度・高精細撮像管であるアバランシェ型ハイビジョンモノクロ新Super-HARPカメラで撮影する。これらの検出器系から高解像度微小血管造影像（50 μm）が得られる[5)-8)]。既存の撮影装置のようにイメージインテンシファイヤーとCCDカメラを用いた検出器では，感度と解像度が低いため，高精細画像として微小血管を描出するには限界がある。

2．病院設置型微小血管造影法

放射光施設は多額のコストと広大な敷地を必要とし，臨床導入するには時間的・空間的にも問題がある。そこで微小血管造影法が臨床応用できるように，新エネルギー・産業技術開発機構（NEDO）の支援により，病院設置型の微小血管造影装置を浜松ホトニクス・NHKエンジニアリングの協力を得て共同開発した。X線管は最大陽極熱容量が5MHUと世界最大級の大きさであるCT用管球を転用した（図❷）。X線高電圧装置も大出力化し，市販の装置では不可能な70kVp・800mAで高輝度のX線を連続20秒間まで撮影できる。疑似単色化はランタノイド系の金属を複合したフィルターで，ヨードのK吸収端である33keV付近に頂点を有し，約20keVのバンド状の照射X線スペクトルに変換した。検出系は，放射光微小血管造影法と同じ，NHKの高感度・高精細撮像管であるアバランシェ型ハイビジョンモノクロ新Super-HARPカメラを用いている。

安全性の検討として，照射X線量と散乱X線量

用語解説

1．**血管再生治療**：虚血性疾患において，血行再建や薬剤治療に抵抗する症例に対し，新生血管を形成させ，血流を改善させる治療である。現在は，自己骨髄やサイトカインなどを用いて臨床応用されている。

2．**微小血管造影法**：既存の血管造影装置の空間解像度は200～300μmであるが，100μm以下の解像度を持つ撮影装置にて微小血管の造影が可能となっている。血管再生治療で再生された血管の評価に期待されている。

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・高エネルギー加速器研究機構
http://www.kek.jp/ja/index.html

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1994年　日本大学医学部卒業後，日本大学第二内科入局
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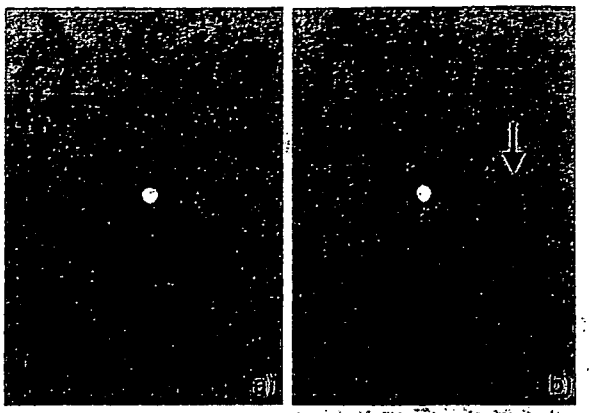
2003年　国立循環器病センター修練医
現在は，大血管疾患から末梢血管疾患の非侵襲的診断法および血管再生治療における微小血管造影法の研究を行っている。

図❺　再生血管のアデノシン投与による反応



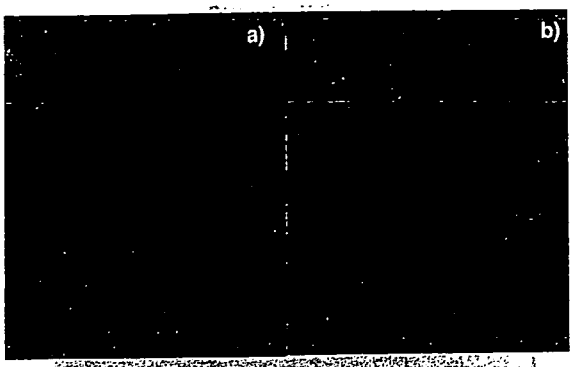
FGF-4-ゼラチンハイドロゲル複合体で治療したウサギ下肢の微小血管造影。アデノシン投与前（a）とアデノシンの投与後（b）。らせん状の血管が拡張するのが観察できる（矢印）。

vascular endothelial growth factor（VEGF）で治療したラットで観察されたらせん状の血管も拡張せず[11]，以上のことから虚血により生じた再生血管やVEGFにより再生したらせん状の再生血管は，内皮機能が備わっていない不完全な再生血管であると考えられた。一方，fibroblast growth factor 4 (FGF-4)-ゼラチンハイドロゲル複合体にて血管再生治療後のウサギの下肢虚血モデルを撮影した。観察されている血管は再生血管と考えられ，アデノシンの投与によりらせん状の血管が拡張したと報告されている[12]。病院設置型の微小血管造影装置でも同様に，FGF-4-ゼラチンハイドロゲル複合体で治療した場合，アデノシンの投与によりらせん状の血管が拡張した（図❺）。これにより，FGF-4-ゼラチンハイドロゲル複合体で治療した場合には，より成熟した血管が再生したと考えられる。

2．病院設置型とプラズマX線微小血管造影装置

病院設置型の微小血管造影装置のX線源では，その単色X線光子数の限界から，現在は体厚が8cmの下肢の微小血管造影に対象が限られている。

図❻　既存のX線装置とプラズマX線装置の比較



イヌ冠動脈ファントムの血管造影。既存のX線装置では心筋貫通枝は観察できないが（a），プラズマX線装置では詳細に観察が可能である（b）。

体厚が8cm程度の被写体では微小血管を描出できるが，10cmを超えると血管像をほとんど得ることができない。一方，プラズマX線装置は，コンデンサーの容量を増加させることで，高輝度化することが可能であるため，人体を通過する疑似単色X線が得られる可能性があると考えられている。マイクロスフィアーを充填したイヌ冠動脈ファントムをプラズマX線装置と既存の血管造影装置とで比較した場合，プラズマX線で撮影した場合は心筋貫通枝レベルのミクロンオーダーの微小血管が詳細に観察できたが，既存の血管造影装置では観察できなかった（図❻）。

おわりに

微小血管造影法にて観察されている血管は，必ずしも新生血管とは限らず，側副血行路の血流の増加やあらかじめ存在していた微小血管の拡張であるかもしれないということを忘れてはならない。しかし，微小血管造影法による画像評価は，血管の種類や反応性の評価まで可能となる。今後の臨床導入により，さらに詳細に検討されることを期待している。