細胞移植と分子イメージング

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1. はじめに

再生医療の諸戦略を筆者なりに整理すると図1のようになる。概念的発端は1993年に報告された，ポリグリコール酸不織布に軟骨細胞を播種した*in vivo*軟骨再生である(図1-②)。産業的には，*in vitro*組織再生(図1-③)が強く望まれ，2007年には，我が国初の細胞組織加工製品である重症熱傷治療用自家培養皮膚製品が承認されるに至った。技術的な完成から長い年月がかかったが，細胞や組織を含む医療機器の幕開けの年となった。スキャホールドと細胞からなる再生医療戦略と比較して，何れかのみを利用する単純な戦略が臨床的には有利である。最も単純と考えられたGTR(図1-①)は，神経誘導管や人工真皮として実現されている。また近年は，様々な周辺科学の発展から，細胞移植療法(図1-④)が実現性の高い再生医療戦略として注目されている。

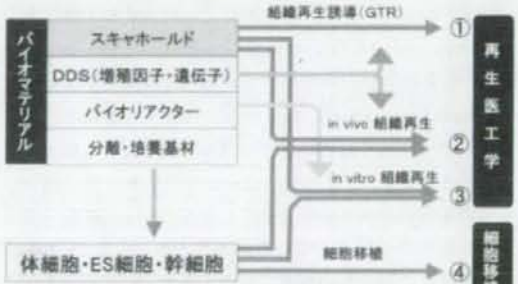

図1 再生医療の戦略

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2. 細胞移植

ES細胞が様々な同種細胞源として大きく期待されているところに，iPS細胞の報告により自己細胞移植医療の可能性が大きく広がった。さらに，ヒト細胞組織加工医薬品等の品質・安全性評価指針「1314号通知」が，近日中に，自己由来製品と同種由来製品に分けて改訂される方針である。その結果，自己細胞の臨床利用が加速すると期待できる。一方で，導入遺伝子の問題やテラトーマなど，まだ課題は残る。

それに対して，骨髄間葉系幹細胞や脂肪由来幹細胞など，患者自身から採取可能な自己細胞の移植医療は，倫理面，制度面におけるハードルが比較的低く，高い実現性を有する再生医療戦略である。

例えば，心疾患に対して，骨格筋芽細胞，脂肪由来幹細胞，間葉系幹細胞などの移植が検討されている。骨髄に存在する間葉系幹細胞は，患者本人からの単離が可能であり，一般的には，骨髄細胞中の接着性細胞として粗精製されて用いられる。Stro-1＋，SH2＋(CD105＋)，CD34－，CD45，CD14－などの表面マーカーの組み合わせによって特定可能であり，その細胞集団は多くのポピュレーションを含んでいる。適切な実験条件において骨，軟骨，脂肪，神経，造血系の細胞，あるいは，血管平滑筋細胞や内皮細胞へ分化することも報告されている。さらに，間葉系幹細胞と心筋細胞を共培養することによって，心筋細胞に特有の表現型を発現すること，また，直接的な細胞同士の相互作用が必須との報告もある[1]。心筋梗塞モデル動物へ移植された間葉系幹細胞は，心筋細胞のマーカーを発現する心筋様細胞への分化が報告されている[2,3]が，自己拍動する心筋様細胞への分化については，未だ，議論中のようだ。このように，様々な分化能をもつ間葉系幹細胞を移植した場合，心筋細

表1　移植細胞の*in vivo*追跡法

モダリティー	ContraTst agent	長所	欠点
光	ルシフェラーゼ基質 赤色蛍光	空間分解能（表層） 感度 時間分解能	小動物に限定 細胞分裂による希釈
SPECT，PET	^{99m}Tc，^{111}Inなど ^{18}F，^{124}Iなど	感度 時間分解能 大動物でも可	放射活性物質の使用 細胞の遺伝子改変
MRI	ランタノイド金属（Gdなど） USPIOなど	空間分解能 大動物でも可	細胞分裂による希釈

胞への分化などの直接的効果が主か，産生する生理活性物質などに起因するパラクライン効果が主かを識別することは極めて困難である。これらを解明するためにも，移植した細胞を非侵襲的に，かつ，長期間にイメージングする技術の開発が必要となる。

3. *In vivo*細胞追跡

非侵襲に移植細胞を*in vivo*イメージングすることは，臨床的な移植効率の最適化（移植数や移植のタイミング，組織生着率，生存期間，機能性の評価）からも非常に重要である。そのためには，移植細胞をレシピエントの細胞と区別する工夫が必要であり，かつ，幹細胞のイメージングに用いるトレーサー・造影剤として，生体適合性，高い安全性，非毒性などの条件を有していなくてはならない。また，遺伝子の変異を起こさないことも重要な要素となる。以下に，近年使用されている3つのイメージング方法について記した[4]（表1）。

1）光イメージング

幹細胞の光イメージングは，現在最も研究が進んでいる分野であり，主に2つの手法が存在する。一つは発光を，もう一つは蛍光を用いる方法である。ルシフェラーゼ遺伝子を導入した細胞を移植後，ルシフェリンを投与することで，生体内で発光させ，近年急速に普及してきた*in vivo*蛍光発光イメージング装置で検出できる[5)~7)]。しかしながら，ルシフェリン発光の波長が約560 nmで組織透過性に劣るため，マウスやラットなどの小動物実験に限られる。近年，組織に関係なく体内を通過できる長波長発光の研究が急速に進んでいる。蛍光イメージングにおいては，長波長蛍光タンパク（700～1,000 nm）やQuantum dotなどが開発されてきた[8)~10)]。

2）SPECT，PETイメージング

Single-photon Emission Computed Tomography（SPECT）やPositron Emission Tomography（PET）を用いた*in vivo*における細胞イメージングは，深部観察も可能で，細胞数などに関してより定量的な議論ができる。この場合，直接トレーサーを細胞内に導入する，または，レセプターを介して細胞へ結合させる手法が取られている[11)~13)]。前者では，長期間，放射線にさらされる問題点がある。レセプター介在型細胞トラッキングでは，移植細胞がレセプターを発現しているかぎり半永久的に細胞の追跡ができ，細胞分裂によってもシグナルは希釈されない。例えば，herpes simplex virus type-1 thymidine kinase（HSV1-TK）を発現させた幹細胞を移植した後に，HSV1-TKの基質である^{18}FHBGを注入することで，移植細胞のイメージングができる[14)]。高感度で長期間，幹細胞の追跡と定量化が可能となる有用な手法である。今後，非特異的なトレーサー分子の取り込み抑制による標的細胞への集積効率の向上が必要とされる。

3）MRIイメージング

3Dイメージングが可能であり，安全性の高いmagnetic resonance imaging（MRI）は*in vivo*における細胞追跡にも有利である。MRIを用いた幹細胞のイメージングでは，T2/T2*による造影が多く用いられる。標的細胞にUltrasmall SuperParamagnetic Iron Oxide（USPIO）を取り込ませる手法が一般的である[6),15),16)]。USPIOの有する磁場は非常に大きいため，細胞の検出に必要な造影剤の数は細胞あたり数千個のオーダーでよい。しかしながら，細胞分裂により細胞内の造影剤濃度が希釈されること，また，幹細胞の崩壊後に造影剤が組織内に残存したり，周囲のマクロファージなどに取り込まれたりするために，長期間の細胞移植追跡には不向きである。

また，Gd^{3+}などのランタノイド系列の金属の利用もある[17)~19)]。一般的な磁場強度で検出するためには，50～500 μMの濃度が必要である。我々のグループでは，主鎖

としてポリビニルアルコールを用い，蛍光物質とGd^{3+}キレートを側鎖に有するMRI用造影剤の開発を行った。ポリビニルアルコールは，その高い親水性のため細胞膜との相互作用が非常に低いことが知られている[20]。そのため，この造影剤を細胞膜内に導入させることで，細胞内に長時間滞在することが可能である。また，ポリビニルアルコールの組織中半減期が非常に短かく，移植細胞が死滅した場合に造影剤が周囲細胞に取り込まれることなく速やかに体外へと排出されるため，生体内で生存している移植細胞のみをイメージングすることが可能である。合成した造影剤を，エレクトロポレーションにより細胞内に送達したところ，ほとんど全ての細胞に造影剤が導入されていた。また，細胞の核ではなく，細胞質に導入されており，細胞から漏洩することなく，細胞内での高い安定性を得ることに成功した。本システムにより，大動物を用いた前臨床研究における細胞移植療法治療効果のメカニズム解明が可能になるのみでなく，有効な移植細胞数を実証することで，最低限のリスクで最大の治療効果を発揮させるための定量的指標を得ることが可能になる。

4. おわりに

それぞれのイメージング法には利点と欠点があるため，近年では，複数の機器を利用したマルチモダリティーイメージングが注目されている。蛍光ラベルを行ったUSPIOを細胞内に導入することで，光，MRイメージングを可能とし[6),15)]，ナノパーティクルを用いることで，MR，超音波，蛍光イメージングが行える[18)]。今後，様々なイメージングに基づいた，新たな現象や治療効果の解明が待たれる。

文　献

1) Nagaya N, Fujii T, Iwase T, et al: Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol **287**: 2670-6, 2004
2) Tuan RS, Boland G, Tuli R: Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Res Ther **5**: 32-45, 2003
3) Minguell JJ, Erices A: Mesenchymal stem cells and the treatment of cardiac disease. Exp Biol Med **231**: 39-49, 2006
4) Frangioni JV, Hajjar RJ: *In vivo* Tracking of stem cells for clinical trials in cardiovascular disease. Circulation **110**: 3378-84, 2004
5) Kim DE, Tsuji K, Kim YR, et al: Neural stem cell transplant survival in brains of mice: Assessing the effect of immunity and ischemia by using real-time bioluminescent imaging. Radiology **241**: 822-30, 2006
6) Li Z, Suzuki Y, Huang M, et al: Comparison of reporter gene and iron particle labeling for tracking fate of human embryonic stem cells and differentiated endothelial cells in living subjects. Stem Cells Published online 2008
7) Tanaka M, Swijnenburg RJ, Gunawan F, et al: *In vivo* Visualization of cardiac allograft rejection and trafficking passenger leukocytes using bioluminescence imaging. Circulation **112**: 1105-10, 2005
8) Michalet X, Pinaud FF, Bentolila LA, et al: Quantum dots for live cells, *in vivo* Imaging, and diagnostics. Science **307**: 538-44, 2005
9) Rosen AB, Kelly DJ, Schuldt AJ, et al: Finding fluorescent needles in the cardiac haystack: tracking human mesenchymal stem cells labeled with quantum dots for quantitative *in vivo* three-dimensional fluorescence Analysis. Stem Cells **25**: 2128-38, 2007
10) Tögel F, Hu Z, Weiss K, et al: Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Physiol Renal Physiol **289**: F31-42, 2005
11) Cao F, Lin S, Xie X, et al: *In vivo* Visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. Circulation **113**: 1005-14, 2006
12) Kang WJ, Kang HJ, Kim HS, et al: Tissue distribution of ^{18}F-FDG-labeled peripheral hematopoietic stem cells after intracoronary administration in patients with myocardial infarction. J Nucl Med **47**: 1295-301, 2006
13) Kraitchman DL, Tatsumi M, Gilson WD, et al: Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. Circulation **112**: 1451-61, 2005
14) Acton PD, Zhou R: Imaging reporter genes for cell tracking with PET and SPECT. Q J Nucl Med Mol Imaging **49**: 349-60, 2005
15) Hinds KA, Hill JM, Shapiro EM, et al: Highly efficient endosomal labeling of progenitor and stem cells with large magnetic particles allows magnetic resonance imaging of single cells. Blood **102**: 867-72, 2003
16) Anderson SA, Glod J, Arbab AS, et al: Noninvasive MR imaging of magnetically labeled stem cells to directly identify neovasculature in a glioma model. Blood **105**: 420-5, 2005
17) Liu M, Guo YM, Wu QF, et al: Paramagnetic particles carried by cell-penetrating peptide tracking of bone marrow mesenchymal stem cells, a research *in vitro*. Biochem Biophys Res Commmun **347**: 133-40, 2006
18) Modo M, Cash D, Mellodew K, et al: Tracking transplanted stem cell migration using bifunctional, contrast agent-enhanced, magnetic resonance imaging. NeuroImage **17**: 803-11, 2002
19) Orlic D, Hill JM, Arai AE: Stem cells for myocardial regeneration. Circ Res **91**: 1092-102, 2002
20) Yamaoka T, Tabata Y, Ikada Y: Comparison of body distribution of poly (vinyl alcohol) with other water-soluble polymers after intravenous administration. J. Pharm. Pharmacol **47**: 479-86, 1995

Original Article

Long-Term Probucol Treatment Prevents Secondary Cardiovascular Events: a Cohort Study of Patients with Heterozygous Familial Hypercholesterolemia in Japan

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Aim: The POSITIVE study assessed whether long-term treatment with probucol, a potent anti-oxidant and cholesteryl ester transfer protein (CETP) activator, is associated with a lowered risk of cardiovascular events in a very high-risk population: familial hypercholesterolemia (FH).
Methods: The study cohort included 410 patients with heterozygous FH, diagnosed between 1984 and 1999 by cardiovascular and metabolic experts at fifteen centers. Traceable patients were screened using predefined eligibility criteria. The primary outcome measure for comparison between probucol exposure and non-exposure was the time to the first cardiovascular event involving hospitalization.
Results: Analysis revealed significant differences in baseline characteristics and follow-up treatment between exposure and non-exposure. An observed indication bias was the use of probucol in more severe FH at diagnosis, both for primary and secondary prevention. When the multivariate Cox regression procedure was used after adjustment for possible confounding factors, probucol lowered the risk (hazard ratio [HR], 0.13; 95% confidence interval [CI], 0.05–0.34) in secondary prevention ($n=74$) and was statistically significant ($p<0.001$), although not significant (HR, 1.5; 95% CI, 0.48–4.67; $p=0.49$) in primary prevention ($n=233$). Safety assessment found no specific difference between exposure and non-exposure.
Conclusion: Long-term probucol treatment may prevent secondary attack in a higher cardiovascular risk population of heterozygous FH.

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Key words; Atherosclerosis, Antioxidants, CETP activator, Dyslipidemia

Introduction

Cardiovascular (CV) diseases, including coronary heart disease and stroke, are the leading cause of death in Japan. Prevention of fatal CV events is therefore the final goal as well as the rationale of cholesterol-lowering therapy.

Probucol, a conventional cholesterol-lowering drug, originated with the report by Barnhart in 1970[1]. The drug has been used clinically in Japan since 1985. Nearly 60,000 Japanese patients still take probucol; western countries discontinued probucol use after

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the original manufacturer's withdrawal notice to the United States FDA in 1995 after 18 year's use of the drug. Probucol's cholesterol-lowering mechanism has not yet been clearly established, but it is thought to increase catabolic excretion of cholesterol into bile[2]. Later studies[3-5] have described new mechanisms of probucol, including anti-atherogenic and anti-oxidant actions. Another controversial and anti-atherogenic feature of probucol is its paradoxical effect of lowering high-density lipoprotein cholesterol (HDL-C). This action reflects, most likely, its molecular mechanisms: promoting cholesterol efflux, and enhancing reverse cholesterol transport by activation of cholesteryl ester transfer protein (CETP)[6-8] and class B type I scavenger receptor[9,10]. Matsuzawa and his colleagues reported an observed close correlation between the extent of regression in Achilles' tendon xanthoma and probucol-induced decrease in HDL-C levels in patients with familial hypercholesterolemia (FH)[11].

No large-scale, randomized, double blind comparative study has been conducted to justify the use of probucol in the prevention of CV events or diseases. however, clinical studies as well as pre-clinical data have been accumulating evidence of the clinical worth of probucol in arteriosclerotic diseases. Numerous clinical results, including a reduction in Achilles' tendon xanthoma thickness after long-term treatment for FH[12, 13], reduced rates of restenosis after angioplasty[14-16], and a decrease in carotid artery intima-media thickness[17, 18] support the therapeutic and preventative effects of probucol on arteriosclerotic lesions and plaque. To evaluate the risk and benefit of long-term probucol treatment, we conducted a cohort study to determine whether probucol treatment is associated with the risk reduction of CV events in patients with heterozygous FH, a very high-risk population.

Methods

Study Cohort

We registered patients with FH who received treatment between January 1, 1984 and December 31, 1999 at 15 centers specializing in CV and metabolic diseases, including FH, nationwide. Patients were traceable by medical recend and met the diagnostic criteria for heterozygous FH under the Japan Atherosclerosis Society Guidelines (2002) for the Diagnosis and Treatment of Atherosclerotic CV Diseases[19]. Definite heterozygous FH was defined as having at least two of the major features: total cholesterol (TC) of 260 mg/dL and above; tendon xanthoma or xanthoma tuberosum; reduced or abnormal receptor activity noted by LDL receptor analysis. Probable heterozygous FH was defined as having at least one each of the major (as above) and minor features: palpebral xanthoma; arcus juvenilis (< 50 years); juvenile (< 50 years) ischemic heart disease. For other eligibility criteria, we excluded patients with possible homozygous FH or with severe ventricular arrhythmias (polymorphic premature ventricular contractions). Possible homozygous FH was defined as having any one of the clinical features: defect of homozygous or hetero-polymeric LDL receptors confirmed by gene analysis; no LDLR activity observed by receptor analysis, severe elevation of plasma TC higher than 500 mg/dL; xanthoma or atherosclerotic vascular lesions including symptoms of juvenile ischemic heart disease; hypercholesterolemia confirmed in both parents; history of ischemic heart disease confirmed in both parents; or poor response to any 3-hydroxy-3methyl-glutaryl-coenzyme A reductase inhibitor (statin).

During the study period between June, 2004 and September, 2005, we collected anonymous case report forms with the patients' baseline data, including medical history, findings at clinical examination, medication data, and laboratory data. The investigators transcribed the data on to case report forms (identified by a code) from the stored medical charts of the patients. The observation period was the period for which each patient's clinical course could be traced. The longest observation period exceeded 20 years for patients on stable doses of probucol.

We required a sample size of 200 in both the probucol exposure and non-exposure groups, supposing a difference of 10% in the incidence of CV events for 5 years (15% in exposure and 25% in non-exposure). A least 400 subjects were needed to detect the difference with 80% power and a type I error of 5% at the 5% significance level with two-sided log-rank test based on normal approximation. The study protocol was approved through the process of ethics committee or institutional review board at each center.

Definitions and Endpoints

The primary outcome measure was the time to the first CV event, defined as acute myocardial infarction (MI), angina pectoris (AP), heart failure (HF), stroke, transient ischemic attack (TIA) or arteriosclerotic peripheral artery diseases (PAD) leading to hospitalization or death as well as sudden death within 24 hours of an observed intrinsic event. The obtained baseline data at the first visit of each patient included demographic characteristics: sex, date of diagnosis at the participant medical center, age, height, weight, and habits of smoking and drinking. Body mass index (BMI) was calculated as weight in kilograms divided

by the square of height in meters. The other collected characteristic factors at diagnosis were the presence of xanthoma and its location, prior CV event, onset date if any prior CV event, treatment for the event, and other possible risk factors for CV events, including the presence of hypertension, diabetes, ventricular arrhythmia, and PAD. We collected data on cholesterol-lowering therapy (with or without probucol) and other concomitant therapy with anti-platelet, antihypertensive or diabetic drugs. Dates of drug initiation, discontinuation, re-administration, and termination were entered as elemental information. Treatment period was defined as the length from initiation until medication termination, or until the occurrence of the defined CV event, whichever came first. A lipid profile of TC, triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and HDL-C, blood pressure, level of fasting blood sugar (FBS), hemoglobinA_{1c} (HbA_{1c}), and thickness of tendon xanthoma in both feet were variables of interest, seen as potential predictors of CV events. We obtained measurements of those variables on a yearly basis after each patient was diagnosed. LDL-C levels were calculated from TC and HDL-C measurements with the Friedewald formula in TG <400 mg/dL. For TG of 400 mg/dL and more than 400 mg/dL, the expression of 0.16 X TG was applied in stead of 0.2 X TG[20]. Most patients had fasted compliantly at periodic checkups of their lipid levels. We set a follow-up period of 10 years for the measurements.

Statistical Analyses

The primary objective of analysis was a comparison between probucol exposure and non-exposure to evaluate whether treatment with probucol (500 mg to 1,000 mg daily) for FH provided CV benefits. The analysis was based on intent-to-treat principles. The secondary objective was to assess whether changes in the lipid profile after probucol treatment predicted CV events in the cohort. Event-free survival, defined as the time from diagnosis to the first CV event, was determined as a response variable. Statistical analysis was performed to evaluate clinical outcomes separately for secondary and primary prevention groups; that is, patients with or without a history of CV events at diagnosis.

Baseline characteristics of each group were explored to detect risk factors for CV events because potential confounders, including indication bias, were anticipated. For baseline comparison, Wilcoxon's rank sum test and Fisher's exact test were used for continuous variables and categorical variables respectively. For detection of risk factors, univariate Cox proportional hazards regression with a baseline variable as covariate was used as a screening step to determine the relationship with CV events. Variables that achieved significance at the level of 20% in univariate analysis were subsequently included in a multivariate Cox proportional hazards regression using backward variable selection. Variables proving significant at the 10% significance level were selected as risk factors to be adjusted. Consequently, probucol treatment effect was evaluated using the multivariate Cox model with adjustment for the selected baseline variables. Finally, the other observed treatment factors: cholesterol-lowering drugs other than probucol, LDL-apheresis, anti-platelet drugs, anti-hypertensive drugs, and diabetic drugs were entered into that model to assess their effects.

For the association between changes in lipid profile after probucol treatment and the risk of CV events, pre-treatment values of TG, LDL-C, HDL-C as well as TC, and each lipid reduction ratio after treatment were used as covariates. Multivariate analyses of time from probucol start to the first CV event used multivariate Cox's proportional hazards models. Statistical analysis was peerformed with SAS version 8.2.

Results

Patient Characteristics

We collected data from the medical records of 541 patients, and excluded the data of 131 patients that did not meet eligibility predefined in the protocol.

The flow diagram (**Fig. 1**) gives reasons for the exclusion. A substantial fraction of probucol-exposed patients, 80.0% and 93.2%, took probucol within two years after diagnosis for in primary and secondary prevention groups, respectively. Baseline characteristics at diagnosis are given for each group (**Table 1**, **2**). The secondary prevention group (**Table 2**) had prior diseases of AP, MI, stroke, HF, and TIA. This group was found to have significant higher proportions of men (60.2%, $p<0.01$), smokers (50.0%, $p<0.01$), hypertension (40.9%, $p<0.001$) diabetes (15.9%, $p=0.02$), and older median age (52 years, $p=0.01$) than the primary prevention group. Moreover, the group tended to have hypo-HDL cholesterolemia of median 42 (20–90) mg/dL, and to receive combined treatments with anti-platelet drugs (56.8%), anti-hypertensive drugs (53.4%), and LDL-apheresis (14.8%).

Comparison between probucol-exposed and non-exposed groups revealed significant differences in some baseline characteristics and treatments, which showed a confounding indication that patients with more severe FH took probucol. For baseline characteristics, the exposed group for primary prevention had more

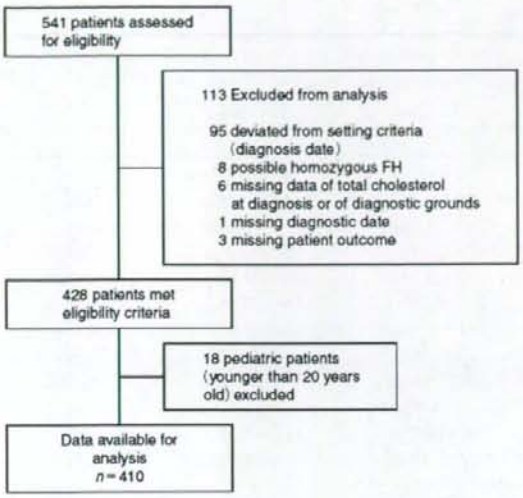


Fig. 1. Patient Flowchart.

We collected data from the medical records of 541 patients, and excluded the data of 131 patients who did not meet the eligibility predefined in the protocol. The flow diagram gives reasons for the exclusion.

palpebral xanthoma (13.4%, $p=0.05$), thicker median measurement of tendon xanthoma (12.5 mm, $p<0.01$), higher median HbA_{1c} (5.8%, $p=0.03$), and more use of antihypertensive drugs (25.3%, $p<0.01$). Their lipid profile was more severe with a higher median baseline TC (325 mg/dL, $p=0.001$), a higher median LDL-C level (253 mg/dL, $p<0.001$), and a lower HDL-C level (47 mg/dL, $p<0.001$) than the unexposed group. The exposed group for secondary prevention had a higher prevalence of post-MI (44.6%, $p<0.01$) than the unexposed group. Observed medications were also significantly different between the exposed and unexposed groups. The exposed group used anti-hypertensive drugs concomitantly at a higher rate (25.3% vs. 11.2%, $p<0.01$) for primary prevention.

Descriptive analysis of baseline characteristics and treatments during observation implies that in both primary and secondary prevention, the exposed groups tended to include patients with more severe FH at diagnosis. Arguably, patients considered more severe at diagnosis would receive more intensive treatment, including probucol.

Outcomes

We present the absolute number of CV events requiring hospitalization by prevention group with details of the events (**Table 3**). The incidence of CV events without consideration of confounding factors was 11.6% in the exposed group and 4.5% in the unexposed group for primary prevention. For secondary prevention, the incidence was 27.0% in the exposed group and 64.3% in the unexposed group. The event-free survival curve of the secondary prevention group is given (**Fig. 2**).

To identify risk factors for CV events, we determined the relationship between the incidence and every baseline variable using univariate Cox regression at a significant level of 20%. Variables proving significant at the 10% significance level in multivariate Cox regression were selected as risk factors to be adjusted. We estimated the effect of treatment after adjusting the selected risk factors. We calculated hazard ratios (HRs) with 95% confidence interval (CI) for binary variables, BMI ≥25 vs BMI <25, drinking vs no drinking, for example, and the indicated HRs corresponded to a 1 standard deviation increase for continuous variables, including TC. Estimated results are given (**Table 4**).

In the primary prevention group, significant variables were BMI ≥25 (HR 1.86, 95% CI 0.87–3.98; $p=0.11$), drinking (HR 2.17, 95% CI 1.02–4.63; $p=0.05$), tendon xanthoma (HR 2.17, 95% CI 0.76–6.23; $p=0.15$), prior diseases other than CV events (HR 1.87, 95% CI 0.87–3.99; $p=0.11$), PAD (HR 5.23, 95% CI 0.70–39.2; $p=0.11$), diabetes (HR 2.27, 95% CI 0.79–6.50; $p=0.13$), TC (HR 1.37, 95% CI 0.99–1.89; $p=0.06$), HDL-C (HR 0.75, 95% CI 0.50–1.12, $p=0.16$), SBP (HR 1.48, 95% CI 1.00–2.18; $p=0.05$), and the thickness of tendon xanthoma (HR 1.50, 95% CI 1.06–2.14; $p=0.02$). Three of these variables, drinking, TC, and PAD were selected for adjustment at the 10% significance level as a result of a multivariate Cox regression with backward variable selection. After adjustment for these three baseline variables, we found no significant effect by probucol at the 5% significant level. The estimated hazard ratio of probucol use for CV events was 1.50 (95% CI 0.48–4.67; $p=0.49$).

In the secondary prevention group, significance variables were drinking (HR 1.74, 95% CI 0.80–3.79; $p=0.17$), presence of palpebral xanthoma (HR 5.34, 95% CI 2.26–12.61, $p<0.001$), TIA (HR 4.16, 95% CI 0.54–32.21; $p=0.17$), history of coronary artery bypass graft (HR 0.31, 95% CI 0.11–0.90; $p=0.03$), hypertension (HR 0.58, 95% CI 0.26–1.28; $p=0.18$), diabetes (HR 2.89, 95% CI 1.30–6.42; $p<0.01$), and fasting blood sugar (HR 1.31, 95% CI 0.91–1.89; $p=0.15$). Two of these variables, palpebral xanthoma and diabetes, were selected for adjustment at the 10% sig-

Table 1. Baseline characteristics of patients in primary prevention group[†]

Characteristics	Primary prevention No. (%) of patients			*p*
	All *n*=322	Exposed *n*=233 (72.4)	Unexposed *n*=89 (27.6)	
Age, mean (range)	49 (27-74)	50 (20-74)	47 (20-72)	0.18
Men, No. (%)	134 (41.6%)	96 (41.2%)	38 (42.7%)	0.90
BMI ≥25	71 (22.5%)	49 (21.4%)	22 (25.6%)	0.45
Smoker	99 (33.2%)	74 (34.1%)	25 (30.9%)	0.68
Drinker	124 (42.2%)	93 (43.7%)	31 (38.3%)	0.43
Xanthoma	259 (80.7%)	190 (81.9%)	69 (77.5%)	0.43
Tendon xanthoma	245 (76.3%)	181 (78.0%)	64 (71.9%)	0.30
Nodular xanthoma	28 (8.7%)	22 (9.5%)	6 (6.7%)	0.51
Palpebral xanthoma	36 (11.2%)	31 (13.4%)	5 (5.6%)	0.05
PAD	4 (1.2%)	1 (0.4%)	3 (3.4%)	0.07
Hypertension	54 (16.8%)	40 (17.2%)	14 (15.7%)	0.87
Diabetes	22 (6.9%)	17 (7.3%)	5 (5.6%)	0.81
Lipid profile, mg/dL				
TC[§]	320 (188-493)	325 (188-493)	307 (194-464)	0.001
TG[§]	120 (28-1289)	121 (34-1068)	120 (28-1289)	0.96
HDL-C[§]	49 (20-108)	47 (20-90)	52 (27-108)	<0.001
LDL-C[§]	244 (45-425)	253 (98-425)	223 (45-403)	<0.001
Blood Pressure, mmHg				
SBP[§]	129 (82-190)	128 (82-190)	131 (90-190)	0.57
DBP[§]	0 (48-120)	80 (48-120)	80 (56-120)	0.91
FBS (mg/dL)[§]	95 (63-276)	94 (63-140)	95 (81-276)	0.41
HbA_{1c} (%)[§]	5.7 (4.1-12.4)	5.8 (4.1-9.7)	5.3 (4.3-12.4)	0.03
Tendon xanthoma thickness (mm)[§]	12.1 (7.5-49.0)	12.5 (7.5-49.0)	10.5 (8.0-20.0)	<0.01
Treatment				
Cholesterol-lowering drugs (non-probucol)	302 (93.8%)	219 (94.0%)	83 (93.3%)	0.80
LDL-apheresis	7 (2.2%)	6 (2.6%)	1 (1.1%)	0.68
Anti-platelet drugs	49 (15.2%)	41 (17.6%)	8 (9.0%)	0.06
Anti-hypertensive drugs	69 (21.4%)	59 (25.3%)	10 (11.2%)	<0.01
Diabetic drugs	15 (4.7%)	12 (5.2%)	3 (3.4%)	0.37

[†]Continuous variables compared by Wilcoxon's rank sum test, distribution of categorical variables by Fisher's exact test. [§]Data are median (range). All data are number (%) unless otherwise indicated. Each percentage shown is related to the total number with measurement data. BMI, body mass index; PAD, peripheral artery disease; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; HbA_{1c}, hemoglobin A_{1c}. LDL-C was calculated with the Friedewald formula.

nificance level as a result of multivariate Cox regression analysis using a backward variable selection. After adjustment for these two baseline variables, the hazard ratio of probucol use for CV events was estimated to be 0.13 (95% CI 0.05-0.34) and significant ($p<0.001$). In sensitivity analyses, we also obtained similar estimation results on probucol for various sets of baseline covariates for adjustment.

The lipid levels of TC, LDL-C and HDL-C were lowered after probucol treatment both in primary and secondary prevention. In the primary prevention group, the median (range) levels of TC, TG, LDL-C and HDL-C closest to before treatment were respectively 305 (165-493), 119 (35-1068), 228 (107-425) and 48 (25-96) mg/dL, and those at 10-year treatment were, respectively, 222 (141-371), 94 (43-335), 157 (91-311) and 39 (17-81) mg/dL. In the secondary prevention, the median levels of TC, TG, LDL-C and HDL-C closest to before atment were, respectively, 320 (191-469), 129 (37-636), 240 (117-381) and 44 (24-90) mg/dL, and those at 10-year treatment were, respectively, 211(135-305), 71 (48-475),

Table 2. Baseline characteristics of patients in secondary prevention group

Characteristics	Secondary prevention No. (%) of patients			p
	All $n=88$	Exposed $n=74$ (84.1)	Unexposed $n=14$ (15.9)	
Age, mean (range)	52 (23-71)	51 (29-70)	53 (23-71)	0.62
Men, No. (%)	53 (60.2%)	46 (62.2%)	7 (50.0%)	0.55
BMI ≥25	21 (25.3%)	17 (24.3%)	4 (30.8%)	0.73
Smoker	42 (50.0%)	38 (53.5%)	4 (30.8%)	0.23
Drinker	39 (46.4%)	33 (46.5%)	6 (46.2%)	1.00
Xanthoma	75 (85.2%)	63 (85.1%)	12 (85.7%)	1.00
Tendon xanthoma	71 (80.7%)	61 (82.4%)	10 (71.4%)	0.46
Nodular xanthoma	7 (8.0%)	6 (8.1%)	1 (7.1%)	1.00
Palpebral xanthoma	8 (9.1%)	5 (6.8%)	3 (21.4%)	0.11
PAD	2 (2.3%)	2 (2.7%)	0 (0.0%)	1.00
Hypertension	36 (40.9%)	30 (40.5%)	6 (42.9%)	1.00
Diabetes	14 (15.9%)	9 (12.2%)	5 (35.7%)	0.04
Lipid profile, (mg/dL)				
TC[†]	332 (191-469)	334 (191-469)	322 (229-444)	0.41
TG[†]	128 (37-636)	128 (37-636)	136 (63-318)	0.85
HDL-C[†]	42 (20-90)	42 (20-90)	39 (26-73)	0.91
LDL-C[†]	249 (117-381)	256 (117-381)	245 (138-354)	0.57
Blood Pressure, mmHg				
SBP[†]	129 (90-180)	128 (96-180)	136 (90-166)	0.97
DBP (mmHg)[†]	80 (52-114)	80 (52-114)	78 (60-104)	0.33
FBS (mg/dL)[†]	96 (72-252)	97 (72-197)	94 (79-252)	0.96
HbA1c (%)[†]	5.8 (4.1-10.6)	5.5 (4.1-8.1)	6.4 (5.3-10.6)	0.06
Tendon xanthoma thickness (mm)[†]	14.5 (5.8-25.0)	15.0 (5.8-25.0)	10.0 (8.5-18.8)	0.09
Prior CV events				
Angina Pectoris	45 (51.1%)	36 (48.6%)	9 (64.3%)	0.39
Myocardial Infarction	34 (38.6%)	33 (44.6%)	1 (7.1%)	<0.01
Stroke	7 (8.0%)	4 (5.4%)	3 (21.4%)	0.08
Heart failure	2 (2.3%)	2 (2.7%)	0 (0.0)	1.00
TIA	2 (2.3%)	1 (1.4%)	1 (7.1%)	0.29
Treatment				0.08
Cholesterol-lowering drugs (non-probucol)	81 (92.0%)	70 (94.6%)	11 (78.6%)	
LDL-apheresis	13 (14.8%)	11 (14.9%)	2 (14.3%)	1.00
Anti-platelet drugs	50 (56.8%)	44 (59.5%)	6 (42.9%)	0.38
Anti-hypertensive drugs	47 (53.4%)	42 (56.8%)	5 (35.7%)	0.24
Diabetic drugs	6 (6.8%)	3 (4.1%)	3 (21.4%)	0.05

[†]Data are the median (range). All data are numbers (%) unless otherwise indicated. Each percentage is related to the total number with measurement data. TIA indicates transient ischemic attack.

147 (124-197) and 33 (17-70) mg/dL. Sub-analysis of changes in the lipid profile after probucol treatment detected significant three predictors of CV event risk: higher baseline TC (HR 2.74, 95% CI 1.05-7.16; $p=0.04$) in the primary prevention group; reduction in TG (HR 0.22, 95% CI 0.06-0.86; $p=0.03$); and reduction in LDL-C (HR 0.17,95% CI 0.03-0.90; $p=0.04$) after treatment in the subset of the secondary prevention group on stable doses of probucol. Neither TC nor HDL-C after treatment was associated with CV event risk in the probucol-exposed group, which indicates that reduction of the HDL-C level after probucol treatment is not related to CV event risk for probucol-exposed patients.

We evaluated the safety of probucol for all collected data from 541 patients, and found 56 adverse

Table 3. Incidence of cardiovascular events

		Cardiovascular Event	No event	Total	p
Primary prevention (n=322)	Exposed (n=233)	27 (11.6%)	206	233	
	MI	4			
	AP	18			
	Str.	3			
	TIA	1			
	PAD	1			0.058
	Unexposed (n=89)	4 (4.5%)	85	89	
	AP	1			
	Str.	2			
	TIA	1			
Secondary prevention (n=88)	Exposed (n=74)	20 (27.0%)	54	74	
	MI	6			
	AP	12			
	HF	1			
	Str.	1			0.012
	Unexposed (n=14)	9 (64.3%)	5	14	
	MI	2			
	AP	6			
	Str.	1			

MI, myocardial infarction; AP, angina pectoris; HF, heart failure; Str., stroke; TIA, transient ischemic attack; PAD, peripheral artery disease.
[†]One of the 4 patients died after 12 months of probucol termination.

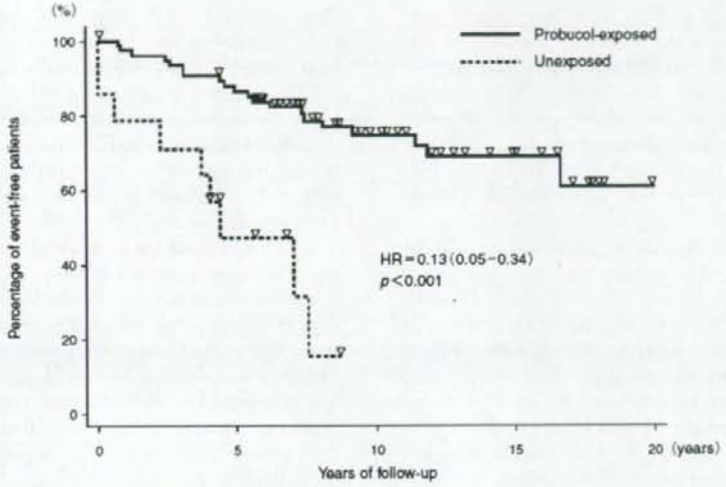
events in 18 patients. Malaise, pruritus, macrocytic anemia and pain in the extremities were recorded as adverse drug reactions associated with probucol. We noted and reported gastric cancer stage III immediately to the Ministry of Health and Welfare as an unexpected serious event, because of an unknown drug relation due to many concomitant drugs, although probucol was found to be non-carcinogenic alone[21]. Six deaths were observed in the population not taking probucol or stopping probucol. There was no other difference in the incidence of adverse events, including serious events, between probucol exposure and non-exposure.

Discussion

Many data from large-scale randomized controlled trials have overwhelmingly demonstrated the clinical benefits of lowering cholesterol with statins[22, 23], yet the rapid and extensive prophylactic use of cholesterol-lowering drugs remains controversial. Few studies have addressed the clinical risks and benefits of long-term treatment of hyperlipidemia among women[24] or elderly patients[25]. The safety of long-term cholesterol-lowering therapy, including the issue of associated cancer risk or benefit, remains inconclusive because of conflicting clinical evidence[26]. More importantly, conclusions from the results of randomized controlled trials are limited by their relatively short follow-up periods (generally less than 5 years) in the analyzed studies.

In long-term treatment for FH, probucol was used with other cholesterol lowering drugs in over 80% of the secondary prevention group–those with a more severe clinical outlook than the primary prevention group: a higher prevalence of hypertension and diabetes, significant thicker tendon xanthoma, more combined therapy with LDL-apheresis, anti-platelet drugs, and anti-hypertensive drugs. The high rate of probucol use in FH was surprising, different from expected. This might partly reflect the prescription behavior of experts with the result that intractable patients responded to the regimen.

In the secondary prevention, the higher-risk group, probucol exposure was associated with a reduction in the risk of cardiovascular events (HR 0.13; 95% CI 0.05–0.34) with high significance ($p<0.001$), while it was not significant in the primary prevention group. This result was also contrary to our expectation that probucol exposure would likely be associated with increased event risk due to a confounding indication–that patients considered more severe at diagnosis would receive more treatment, including probucol. We did not collect the details of non-probucol drugs



Number at risk

Years	0					5					10					15					20
Exposed	74	71	70	68	66	62	54	50	42	38	34	30	25	19	17	13	12	8	3	1	0
Unexposed	14	11	11	10	9	5	4	2	1	0	0	0	0	0	0	0	0	0	0	0	0

Estimates of event-free rates are according to whether patients received probucol. The cumulative probability of remaining without events was higher in patients treated with probucol ($p<0.001$; log-rank test).

Fig. 2. Kaplan-Meier Estimates of Event-free Rate.

For secondary prevention, the incidence of cardiovascular events was 27.0% in the exposed group and 64.3% in the unexposed group. An event-free survival curve for the secondary prevention group is given.

Table 4. The results of multivariate analysis using Cox regression procedure

Factor	Primary prevention			Secondary prevention		
	HR	95% CI	*p*	HR	95% CI	*p*
Baseline variables						
Total cholesterol	1.58	1.06-2.33	0.02	-	-	-
Drinking	2.43	1.09-5.44	0.03	-	-	-
Peripheral artery disease	5.27	0.51-54.63	0.16	-	-	-
Palpebral xanthoma	-	-	-	2.94	1.02-8.47	0.05
Diabetes	-	-	-	2.58	0.76-8.76	0.13
Treatment in follow-up						
Probucol use	1.50	0.48-4.67	0.49	0.13	0.05-0.34	<0.001
Anti-platelet drug use	-	-	-	2.48	1.00-6.17	0.05

to simplify the study procedure. However, we would likely exclude underused statins because of the reduced use of non-probucol drugs from the possible factors of the higher event rate in the unexposed group, because statins were available when all of the 9 recurrent patients (**Table 3**) started and the patients continued on cholesterol-lowering drugs. We suppose, therefore, that the reasons for this unanticipated great risk reduction include some antioxidant and anti-atherogenic actions[3, 4, 27] of probucol. The finding in second prevention may be suggested by the report[27] that probucol significantly decreased *in vitro* LDL oxidizability measured under typically strong oxidative conditions, and that long-term treatment with probucol had an

anti-atherogenic effect in Watanabe Heritable Hyperlipidemic rabbits. From the observation that the baseline lipid profile was not different between the two groups of exposure and non-exposure in secondary prevention, the drug might exhibit greates effectiveness in post-cardiovascular disease patients, in possibly advanced lipid accumulation and inflammation, which are associated with the circulation of oxidized LDL[28].

In primary prevention, we observed an almost significant increase of events in the exposed group (**Table 3**), and an apparently increased risk (HR 1.5), although not statistically significant after adjustment (**Table 4**). We suppose, however, that the ideal effects of probucol might be concealed by the following factors noted in primary prevention. The exposed group had a worse lipid profile (TC, LDL-C and HDL-C levels), higher HbA1c, and thus definitely a higher risk than the unexposed group. Furthermore, 8 (nearly 30%) of the 27 patients experiencing cardiovascular events in the exposed group discontinued probucol when they had events. This was consistent with the different finding between primary and secondary preventions in the exposed group: less than half of the patients (113 of 233) in primary prevention continued on probucol, while 53 (72%) of 74 patients continued in secondary prevention. This estimation might be conservative.

The controversial and paradoxical action of probucol– lowering HDL-C– level was not associated with the risk of CV events in the cohort, therefore, the association between low levels of HDL-C and an increased risk for CV events or death indicated by the early Framingham Heart Study[29] may not be extrapolated to probucol-treated patients. This proposition is consistent with recent findings that a lowered HDL-C level is not always atherogenic, but that the quality or function of HDL-C is more important than the HDL-C levels[30]. In fact, increased levels of HDL-C with torcetrapib, a CETP inhibitor, were not associated with a significant clinical benefit in patients with coronary disease[31], FH[32] or mixed dyslipidemia[33].

We speculate that enhanced reverse cholesterol transport by CETP activation as a result of probucol treatment also contributed to the detected risk reduction in the cohort. The observed positive outcome of probucol, a CETP activator, might be a mirror image of the negative clinical trial results for the CETP inhibitor[34]. Reports[35, 36] of increased coronary heart disease in CETP deficiency despite increased HDL-C levels, and the molecular approach to review CETP deficiency[37] support our hypothesis, at least in Japanese genealogy. Interestingly, a recent basic research reports that human CETP expression enhances the mouse survival rate in an experimental systemic inflammation model[38], indicating for the first time a role for CETP in the defense against the exacerbated production of proinflammatory mediators.

For the safety evaluation, we found no cardiotoxic adverse drug reaction including QT/QTc prolongation or torsade de pointes, in this study, although probucol can cause them[16, 39, 40].

We obtained these results from an observational study with no control for inaccuracy, unexpected bias or confounding factors. We could not assure the precision of the baseline measurements due to unrecorded data. The participant centers were major hospitals for FH, but not all hospitals in Japan, because the study was conducted as part of a post-marketing study by a pharmaceutical manufacturer within the framework of the Japanese government regulations. Some restrictions on collecting data might have resulted in unexpected small numbers in the unexposed group in secondary prevention, although we think that the study cohort represents nearly a nationwide population of heterozygous FH in Japan. The results derived from patient data in Japan can not necessarily be generalized to patients in western countries.

Despite these limitations of the study, however, we could evaluate the outcome of long-term probucol treatment in the medical practice setting for FH, a high-risk population, for as long as 20 years in Japan. The significant risk reduction of CV events observed in the secondary prevention group holds clinical significance and suggests some beneficial therapeutic actions of this drug in arteriosclerotic diseases. The hypothesis from the findings warrants a randomized controlled trial for verification of the secondary prevention, and needs further research into the molecular mechanisms or roles of CETP in pathogenesis.

Author Contributions

Dr. Yamashita had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Matsuzawa, Kita, Saito, Fukushima, Matsui. Acquisition of data: Yamashita, Bujo, Arai, Harada-Shiba, Saito, Kita, Matsuzawa. Analysis and interpretation of data: Yamashita, Bujo, Arai, Harada-Shiba, Matsui, Saito, Fukushima, Kita, Matsuzawa.

Drafting of the manuscript: Yamashita, Bujo, Arai, Harada-Shiba, Matsui, and Fukushima. Critical revision of the manuscript for important intellectual content: Yamashita, Matsui, Fukushima, Kita, Saito,

and Matsuzawa. Statistical analysis: Matsui and Fukushima. Administrative, technical, or material support: Fukushima, Matsui, Kita, Saito, and Matsuzawa. Study supervision: Yamashita, Fukushima, Matsui, Kita, Saito, and Matsuzawa.

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Disclosures

From the formerly Daiichi and Otsuka, Dr. Matsui, Dr. Fukushima, Dr. Matsuzawa, and Dr. Kita received fees and expenses for meetings related to protocol design, statistical and clinical interpretation of the data; Dr. Bujo, Dr. Arai, Dr. Harada-Shiba received honoraria and travel expenses for lectures, Dr.Yamashita, Dr. Bujo, Dr. Arai received fees and travel expenses for a meeting related to clinical interpretation of the data. Dr. Yamashita received consultancy fees from Otsuka. Dr. Matsuzawa is contracted as a short-term adviser to Otsuka in medical science. Dr. Saito received travel expenses only.

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References

1) Barnhart JW, Sefranka JA, McIntosh DD: Hypocholesterolemic effect of 4,4'-(isopropylidenedithio)-bis (2,6-di-t-butylphenol) (probucol). Am J Clin Nutr. 1970; 23:1229-1233
2) Tawara K, Tomikawa M, Abiko Y: Mode of action of probucol in reducing serum cholesterol in mice. Jpn J Pharmacol. 1986; 40:123-133
3) Kita T, Nagano Y, Yokode M, Ishii K, Kume N, Ooshima A, Yoshida H, Kawai C: Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. Proc Natl Acad Sci USA. 1987; 84:5928-5931
4) Carew TE, Schwenke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci USA. 1987; 84:7725-7729
5) Siveski-Iliskovic N, Kaul N, Singal PK: Probucol promotes endogenous antioxidants and provides protection against adriamycin-induced cardiomyopathy in rats. Circulation. 1994; 89:2829-2835
6) McPherson R, Hogue M, Milne RW, Tall AR, Marcel YL: Increase in plasma cholesteryl ester transfer protein during probucol treatment. Relation to changes in high density lipoprotein composition. Arterioscler Thromb. 1991; 11:476-481
7) Chiesa G, Michelagnoli S, Cassinotti M, Gianfranceschi G, Werba JP, Pazzucconi F, Sirtori CR, Franceschini G: Mechanisms of high-density lipoprotein reduction after probucol treatment: changes in plasma cholesterol esterification/transfer and lipase activities. Metabolism. 1993; 42:229-235
8) Ishigami M, Yamashita S, Sakai N, Hirano K, Arai T, Maruyama T, Takami S, Koyama M, Kameda-Takemura K, Matsuzawa Y: High-density lipoproteins from probucol-treated patients have increased capacity to promote cholesterol efflux from mouse peritoneal macrophages loaded with acetylated low-density lipoproteins. Eur J Clin Invest. 1997; 27:285-292
9) Rinninger F, Wang N, Ramakrishnan R, Jiang XC, Tall AR: Probucol enhances selective uptake of HDL-associated cholesteryl esters in vitro by a scavenger receptor B-I-dependent mechanism. Arterioscler Thromb Vasc Biol. 1999; 19:1325-1332
10) Hirano K, Ikegami C, Tsujii K, Zhang Z, Matsuura F, Nakagawa-Toyama Y, Koseki M, Masuda D, Maruyama T, Shimomura I, Ueda Y, Yamashita S: Probucol enhances the expression of human hepatic scavenger receptor class B type 1, possibly through a species-specific mechanism. Arterioscler Thromb Vasc Biol. 2005; 25:2422-2427
11) Matsuzawa Y, Yamashita S, Funahashi T, Yamamoto A, Tarui S: Selective reduction of cholesterol in HDL2 frac-

tion by probucol in familial hypercholesterolemia and hyper HDL2 cholesterolemia with abnormal cholesteryl ester transfer. Am J Cardiol, 1988; 62:66B-72B
12) Yamamoto A, Matsuzawa Y, Yokoyama S, Funahashi T, Yamamura T, Kishino B: Effects of probucol on xanthomata regression in familial hypercholesterolemia. Am J Cardiol, 1986; 57:29H-35H
13) Shinomiya M, Nishide T, Tashiro J, Shirai K, Saito Y, Yoshida S: Effect of 5-year administration of probucol on development of myocardial infarction in heterozygous familial hypercholesterolemia. Current Ther Res, 1993; 54:142-151
14) Daida H, Kuwabara Y, Yokoi H, Nishikawa H, Takatsu F, Nakata Y, Kutsumi Y, Oshima S, Nishiyama S, Ishiwata S, Kato K, Nishimura S, Miyauchi K, Kanoh T, Yamaguchi H: Effect of probucol on repeat revascularization rate after percutaneous transluminal coronary angioplasty (from the Probucol Angioplasty Restenosis Trial [PART]). Am J Cardiol, 2000; 86:550-552, A9
15) Tardif JC, Côté G, Lespérance J, Bourassa M, Lambert J, Doucet S, Bilodeau L, Nattel S, de Guise P: Probucol and multivitamins in the prevention of restenosis after coronary angioplasty. Multivitamins and Probucol Study Group. N Engl J Med, 1997; 337:365-372
16) Tardif JC, Grégoire J, Schwartz L, Title L, Laramée L, Reeves F, Lespérance J, Bourassa MG, L'Allier PL, Glass M, Lambert J, Guertin MC; Canadian Antioxidant Restenosis Trial (CART-1) Investigators: Effects of AGI-1067 and probucol after percutaneous coronary interventions. Circulation, 2003; 107:552-558
17) Baldassarre D, Franceschini G, Peruzzotti G, Brusoni B, Sirtori CR: Clinical evaluation of probucol in hypercholesterolemia: individual lipoprotein responses and inhibitory effect on carotid atherosclerosis progression. J Cardiovasc Pharmacol, 1997; 30:784-789
18) Sawayama Y, Shimizu C, Maeda N, Tatsukawa M, Kinukawa N, Koyanagi S, Kashiwagi S, Hayashi J: Effects of probucol and pravastatin on common carotid atherosclerosis in patients with asymptomatic hypercholesterolemia. Fukuoka Atherosclerosis Trial (FAST). J Am Coll Cardiol, 2002; 39:610-616
19) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirobe K, Funahashi T, Yokote K, Yokode M: Committee for Epidemiology and Clinical Management of Atherosclerosis: Primary hyperlipidemia. J Atheroscler Thromb, 2008; 15:49-51
20) DeLong DM, DeLong ER, Wood PD, Lippel K, Rifkind BM: A comparison of methods for the estimation of plasma low- and very low-density lipoprotein cholesterol. The Lipid Research Clinics Prevalence Study. JAMA, 1986; 256:2372-2377
21) Newman TB, Hulley SB: Carcinogenicity of lipid-lowering drugs. JAMA, 1996; 275:55-60
22) Cholesterol Treatment Trialists' (CTT) Collaborators: Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90056 participants in 14 randomised trilas of statins. Lancet, 2005; 366:1267-1278
23) Nakamura H, Arakawa K, Itakura H, Kitabatake A, Goto Y, Toyota T, Nakaya N, Nishimoto S, Muranaka M, Yamamoto A, Mizuno K, Ohashi Y; MEGA Study Group: Primary prevention of cardiovascular disease with pravastatin in Japan (MEGA Study): a prospective randomised controlled trial. Lancet, 2006; 368:1155-1163
24) Walsh JM, Pignone M: Drug treatment of hyperlipidemia in women. JAMA, 2004; 291:2243-2252
25) Ali R, Alexander KP: Statins for the primary prevention of cardiovascular events in older adults: a review of the evidence. Am J Geriatr Pharmacother, 2007; 5:52-63
26) Bonovas S, Filioussi K, Tsavaris N, Sitaras NM: Statins and cancer risk: a literature-based meta-analysis and meta-regression analysis of 35 randomized controlled trials. J Clin Oncol, 2006; 24:4808-4817
27) Bräsen JH, Koenig K, Bach H, Kontush A, Heinle H, Witting PK, Ylä-Herttuala S, Stocker R, Beisiegel U: Comparison of the effects of alpha-tocopherol, ubiquinone-10 and probucol at therapeutic doses on atherosclerosis in WHHL rabbits. Atherosclerosis, 2002; 163:249-259
28) Tsimikas S, Brilakis ES, Miller ER, McConnell JP, Lennon RJ, Kornman KS, Witztum JL, Berger PB: Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease. N Engl J Med, 2005; 353:46-57
29) Wilson PW, Abbott RD, Castelli WP: High density lipoprotein cholesterol and mortality. The Framingham Heart Study. Arteriosclerosis, 1988; 8:737-741
30) Navab M, Anantharamaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fogelman AM: Mechanisms of disease: proatherogenic HDL--an evolving field. Nat Clin Pract Endocrinol Metab, 2006; 2:504-511
31) Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM; ILLUSTRATE Investigators. Effect of torcetrapib on the progression of coronary atherosclerosis. N Engl J Med, 2007; 356:1304-1316
32) Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT, Bots ML; RADIANCE 1 Investigators. Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. N Engl J Med, 2007; 356:1620-1630
33) Bots ML, Visseren FL, Evans GW, Riley WA, Revkin JH, Tegeler CH, Shear CL, Duggan WT, Vicari RM, Grobbee DE, Kastelein JJ; RADIANCE 2 investigators. Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomized, double-blind trial. Lancet, 2007; 370:153-160
34) Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B; ILLUMINATE Investigators: Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med, 2007; 357:2109-2122
35) Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, Curb JD, Tall AR: Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. J Clin Invest 1996; 97:2917-2923
36) Hirano K, Yamashita S, Nakajima N, Arai T, Maruyama T, Yoshida Y, Ishigami M, Sakai N, Kameda-Takemura K,

Matsuzawa Y: Genetic cholesteryl ester transfer protein deficiency is extremely frequent in the Omagari area of Japan. Marked hyperalphalipoproteinemia caused by CETP gene mutation is not associated with longevity. Arterioscler Thromb Vasc Biol. 1997; 17:1053-1059

37) Nagano M, Yamashita S, Hirano K, Takano M, Maruyama T, Ishihara M, Sagehashi Y, Kujiraoka T, Tanaka K, Hattori H, Sakai N, Nakajima N, Egashira T, Matsuzawa Y: Molecular mechanisms of cholesteryl ester transfer protein deficiency in Japanese. J Atheroscler Thromb. 2004; 11:110-121

38) Cazita PM, Barbeiro DF, Moretti AI, Quintão EC, Soriano FG: Human cholesteryl ester transfer protein expression enhances the mouse survival rate in an experimental systemic inflammation model: a novel role for CETP. Shock. 2008; 30:590-595

39) Viskin S, Justo D, Halkin A, Zeltser D: Long QT syndrome caused by noncardiac drugs. Prog Cardiovasc Dis. 2003; 45:415-427

40) Guo J, Massaeli H, Li W, Xu J, Luo T, Shaw J, Kirshenbaum LA, Zhang S: Identification of IKr and its trafficking disruption induced by probucol in cultured neonatal rat cardiomyocytes. J Pharmacol Exp Ther. 2007; 321:911-920

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ORIGINAL ARTICLE

Absolute quantitation of myocardial blood flow with ^{201}Tl and dynamic SPECT in canine: optimisation and validation of kinetic modelling

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Abstract
Purpose ^{201}Tl has been extensively used for myocardial perfusion and viability assessment. Unlike ^{99m}Tc-labelled agents, such as ^{99m}Tc-sestamibi and ^{99m}Tc-tetrofosmine, the regional concentration of ^{201}Tl varies with time. This study is intended to validate a kinetic modelling approach for in vivo quantitative estimation of regional myocardial blood flow (MBF) and volume of distribution of ^{201}Tl using dynamic SPECT.
Methods Dynamic SPECT was carried out on 20 normal canines after the intravenous administration of ^{201}Tl using a commercial SPECT system. Seven animals were studied at rest, nine during adenosine infusion, and four after beta-blocker administration. Quantitative images were reconstructed with a previously validated technique, employing OS-EM with attenuation-correction, and transmission-dependent convolution subtraction scatter correction. Measured regional time–activity curves in myocardial segments were fitted to two- and three-compartment models. Regional MBF was defined as the influx rate constant (K_1) with corrections for the partial volume effect, haematocrit and limited first-pass extraction fraction, and was compared with that determined from radio-labelled microspheres experiments.
Results Regional time–activity curves responded well to pharmacological stress. Quantitative MBF values were higher with adenosine and decreased after beta-blocker compared to a resting condition. MBFs obtained with SPECT (MBF_{SPECT}) correlated well with the MBF values obtained by the radio-labelled microspheres (MBF_{MS}) ($MBF_{SPECT}=-0.067+1.042\times MBF_{MS}$, $p<0.001$). The three-compartment model provided better fit than the two-compartment model, but the difference in MBF values between the two methods was small and could be accounted for with a simple linear regression.
Conclusion Absolute quantitation of regional MBF, for a wide physiological flow range, appears to be feasible using ^{201}Tl and dynamic SPECT.

Keywords Myocardial blood flow · Dynamic SPECT · Thallium-201 · Compartment model · Quantitation

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Introduction

Myocardial perfusion imaging using Thallium-201 (^{201}Tl) is well established in routine clinical practice for detecting

exercise-induced myocardial ischaemia and/or for assessing myocardial viability in patients with coronary artery disease. The diagnosis, however, has been limited to qualitative or visual assessment of the physical extent of the defect areas rather than quantitative assessment of physiological functions. Quantitative methods would for example enable longitudinal studies when assessing therapy response and pharmacological interventions. Some groups have already investigated the feasibility of estimating quantitative parameters with dynamic SPECT in the myocardium using ^{201}Tl [1] and ^{99m}Tc-Teboroxime [1, 2], but these techniques have not yet been applied to clinical practice. This is largely attributed to the fact that quantitative reconstruction programmes are not readily available on commercial SPECT systems.

We have developed a reconstruction programme package for SPECT, which can accurately provide quantitative images of radio-labelled tracer distributions in vivo, which is a pre-requisite for absolute physiological parameter estimation. The adequacy and accuracy of these methods have been demonstrated in multiple papers for ^{99m}Tc and ^{201}Tl in cardiac studies [3–5], and for ^{99m}Tc and ^{123}I in brain studies [6]. It has also been demonstrated, in brain studies, that physiological parameters such as cerebral perfusion [6] and cerebral flow reactivity [7] obtained using our package were as accurate as those determined by PET. These findings suggest that absolute quantitation of regional myocardial perfusion might also be possible in a clinical setting using commercial SPECT cameras.

^{201}Tl is a potassium analogue, and its kinetics has been extensively investigated in previous studies [8, 9]. Due to the high first-pass extraction fraction (EF) [10] and a large distribution volume, ^{201}Tl has been considered an ideal tracer for quantitation of absolute myocardial blood flow, not only at rest but also at hyperemic conditions. As a clinical implication, quantitative assessment of MBF and coronary flow reserve is important. For instance, coronary microvascular dysfunction or impaired endothelial function in patients with coronary risk factors or patients with cardiomyopathy or with heart failure is an un-resolved important issue to answer [11]. Coronary flow reserve can also be reduced in patients with hyper-cholesterolemia without overt coronary stenosis [12]. The low energy and long half-life of ^{201}Tl have, however, seriously limited its use in nuclear cardiology.

The goal of this study was to validate our reconstruction methodology for the estimation of myocardial blood flow using ^{201}Tl and dynamic SPECT using tissue time–activity curves (TTAC) derived from myocardial regions. In addition, we aimed to find the optimal kinetic model configuration and to investigate the factors affecting the estimation of physiological parameters such as the partial volume effect (PVE), appropriate choice of input function, conversion from plasma to blood flow using haematocrit (Hct) and the limited first-pass tracer EF.

Materials and methods

Subjects

A total of 21 dogs were studied in which 8 were in a resting condition, 9 dogs during constant infusion of adenosine for increased MBF, and 4 dogs during constant infusion of beta-blocker. Of the 21 studies, 1 study was un-successful and projection data could not be retrieved from the scanner, reducing the number of resting studies to 7 and total dog studies to 20. Adenosine was infused continuously over the study duration at a rate ranging from 140 to 700 mg/kg/h to achieve a range of blood flow increases. An initial dose of beta-blockers ranging from 2 to 6 mg was given, followed by a constant infusion for the duration of the study of 2 or 4 mg/h. The study protocol was approved by the animal ethics committee at the Akita Research Institute of Brain, Akita City, Japan where all experiments were carried out.

SPECT procedures

All dogs were anaesthetised, and the catheters for dose administration and arterial blood sampling were inserted before the study. The SPECT system was a conventional dual-head gamma camera (Toshiba GCA-7200A, Tokyo, Japan) fitted with short focal length fan-beam collimators (LEHR-Fan). The transverse field-of-view (FOV) was 22 cm diameter and axial FOV was 20 cm. The dogs were carefully taped into a cradle to minimise motion during the study, and also to ensure that no truncation occurred. Heart rate and blood pressure were monitored throughout the study and recorded at regular intervals.

Before the injection of any tracer, a 15-min transmission study was carried out in which a rod source filled with approximately 740 MBq of ^{99m}Tc was placed along the focal line of one of the fan-beam collimators (see Fig. 1). The transmission study was followed by injection of 3 MBq of ^{141}Ce microspheres into the left ventricle via a catheter and blood was withdrawn from the aorta at a constant flow rate of 5 ml/min for 2 min to serve as an input function. For the pharmacological intervention studies, adenosine infusion or beta-blocker injection followed by infusion was commenced before the ^{141}Ce microsphere administration.

Dynamic SPECT was commenced with the start of the 4-min constant infusion of 110 MBq ^{201}Tl. The frame collection rates and 360° rotation times were 10×1 min (rotation time 15 s), 6×2 min (30 s), 3×4 min (60 s) and 5×5 min (60 s) for the first hour for all studies. Resting blood flow studies had an additional 18×10 min (120 s) frames collected for a total study period over 4 h. The shorter total study time for the drug infusion studies was mandated by the difficulties in keeping the dogs stable with prolonged infusions of the drugs used. A 34% energy

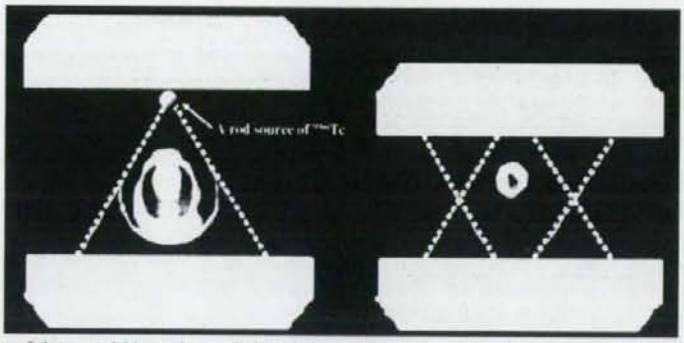

Fig. 1 Schematic diagram of data acquisition using a clinical dual-headed SPECT camera fitted with fan-beam collimators. Transmission scan was performed using a ^{99m}Tc-filled rod source placed at a focal line of one of the collimators, and only one of the detectors was used (*left*). Both detectors were used in the emission scan (*right*)

window centred on 77 keV was used for the ^{201}Tl acquisitions [4, 13].

Arterial blood samples were taken every 20 s for the first 6 min, every 60 s for 6–10 min, 120 s for 10–20 min, 300 s for 20–30 min and 600 s for 30–60 min. For the resting studies, blood samples were also taken every 20 min for 1–2 h and additional samples at 2.5, 3 and 4 h post-^{201}Tl infusion. In six studies, plasma was separated immediately after sampling by centrifugation, and plasma samples were counted in a well counter cross-calibrated with the SPECT scanner. To minimise the effects of the continued exchange of ^{201}Tl between plasma and red blood cells in the test tubes after sampling, immediate, rapid separation of plasma from whole blood was required. An averaged relationship between plasma and whole blood concentration ratio over time was obtained, and then multiplied with the whole blood curves for all studies to derive a plasma input function.

At the end of the SPECT study, the microsphere blood flow measurement was repeated with ^{51}Cr microspheres. The dogs were then killed by injection of potassium chloride (KCl) and the myocardium was dissected into samples suitable for counting in the well counter. The ^{201}Tl concentration in the tissue samples was derived from the sample weight normalised gamma counter counts. The samples were stored to allow for the decay of ^{201}Tl ($T_{1/2}$= 73 h vs $T_{1/2}$=32.5 days for ^{141}Ce and 27.8 days for ^{51}Cr) and then counted to measure the ^{141}Ce and ^{51}Cr activities. Separation between ^{141}Ce and ^{51}Cr counts was based on their respective gamma ray energies (145 keV for ^{141}Ce and 323 keV for ^{51}Cr).

SPECT data processing

Projection data were processed according to previously described procedures [5]. Briefly, the transmission data obtained by the fan-beam collimator were first re-binned into parallel projections. Transmission projections were normalised by blank projection, re-constructed to generate quantitative maps of the attenuation coefficient for ^{99m}Tc and then linearly scaled to provide attenuation correction maps for ^{201}Tl. Emission data were corrected for detector non-uniformity and also re-binned into parallel projections. The projection data were then corrected for scatter with transmission-dependent convolution subtraction (TDCS) originally proposed by Meikle et al. [14] and further optimised by our group [4, 5]. The emission projection data were re-constructed with the OS-EM reconstruction algorithm [15] using three iterations and ten subsets. The re-constructed images were cross-calibrated with the well counter system.

Data analysis

Re-constructed images were normalised by acquisition time for each frame. Multiple circular regions of interest (ROI) were drawn on the myocardium, and the TTAC of ^{201}Tl were generated for the anterior, apical, lateral, posterior and septal areas of the myocardium. The two-compartment model (one tissue compartment) and three-compartment model (two tissue compartments) shown in Fig. 2 were applied to determine two parameters (K_1 and K_2) for the two-compartment model and four parameters (K_1–K_4) for the three-compartment model by means of non-linear least squares fitting (NLLSF).

The regional MBF was considered to be related to K_1 obtained from compartment model fits. K_1 is, however, affected by the PVE, Hct and the limited first-pass EF whose effects were corrected according to Eq. 1:

$$\mathrm{MBF} = \frac{\mathrm{PVE}}{\mathrm{EF} \times (1 - \mathrm{Hct})} \times K_1 \qquad (1)$$

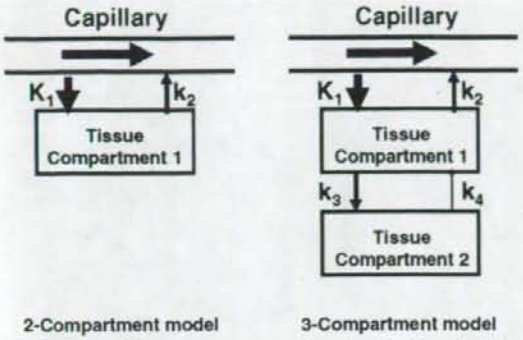

Fig. 2 Two- and three-compartment models evaluated in this study. K_1 in units of ml/min/g denotes the regional MBF for both models. Distribution volume (V_d) in units of ml/g is defined as K_1/k_2 for the two-compartment mode, and $\frac{K_1}{k_2}\left(1+\frac{k_3}{k_4}\right)$ for the three-compartment model

The physiological basis for the correction factors in Eq. 1 can be described as follows:

1. TTACs obtained from SPECT images are under-estimated due to the limited spatial resolution relative to the myocardial wall thickness and also due the myocardial contractile motion. This phenomenon is known as PVE. The PVE correction factor for each TTAC was determined from the ratio of the last SPECT frame counts to the ^{201}Tl myocardial tissue sample counts obtained from the tissue samples taken and measured with the well counter at the end of the SPECT scan.
2. The arterial input function for the compartment model studies was defined from the plasma radioactivity concentration curve, rather than the whole blood radioactivity curve. K_1 is therefore the regional "plasma" flow. Thus, for comparison with the microsphere flow measurements, which estimates the whole blood flow, K_1 was divided by (1−Hct) to obtain the flow for the total blood.
3. For a tracer with limited first-pass EF<1.0, flow (MBF) is related to K_1 by K_1=EF×MBF. The first-pass EF is flow-dependent and decreases at high flow. We have applied an empirical formulation for the first-pass EF based on the data by Weich et al. [10] (EF=0.84−0.524·$\log_{10}(K_1^*)$ where K_1^* is $K_1/(1-\text{Hct})$. The K_1 values obtained with two- and three-compartment models with/without corrections according to Eq. 1 were compared to the average of microsphere blood flow values obtained pre- and post-dynamic SPECT scan.

The distribution volume of ^{201}Tl (V_d) was defined as

$$V_d = \frac{K_1}{k_2} \text{ for the two} - \text{compartment model} \quad (2a)$$

$$V_d = \frac{K_1}{K_2}\left(1+\frac{K_3}{K_4}\right) \text{ for the three} - \text{compartment model.} \quad (2b)$$

As mentioned before, the resting studies were collected for 4 h, whilst the adenosine and beta-blocker studies were collected for approximately 1 h. To investigate whether the shorter collection time introduces systematic bias, NLLSF fits restricted to the first 1 h of the resting study data were also performed and compared with the V_d values from the full 4 h resting data set and with the estimates obtained from the beta-blocker and adenosine studies.

Akaike information criterion (AIC) and Schwarz criterion (SC) were calculated for both two-compartment and three-compartment model fits [16] to test the adequacy of the two models. All data are presented as mean+1 SD. Student's t test was employed in the comparison of the V_d values. Pearson's regression analysis was applied to compare K_1 and microsphere flow values. A probability value of <0.05 was considered statistically significant.

Results

Figure 3 shows the plasma to whole blood concentration ratios in the six dogs with rapid plasma separation and the averaged data. Equilibrium is reached after about 40 min, at which time the mean ratio was found to be 0.76. As expected, relative plasma concentration is highest early on as the tracer is injected into the plasma (and not red blood cells). ^{201}Tl is rapidly cleared from the plasma causing a rapid decline in relative plasma concentration and "undershoot" before equilibrium is established. Samples left for a prolonged period before plasma separation showed the value of approximately 0.78, which was close to the plasma to whole blood concentrations ratio at the equilibrium shown in

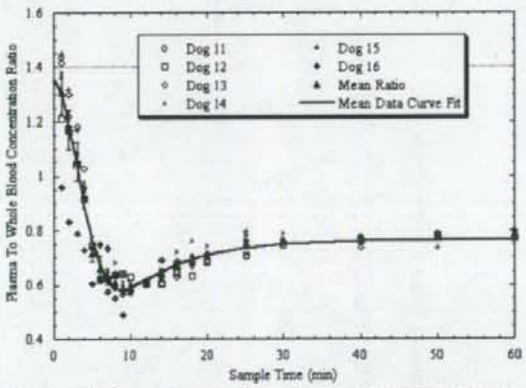

Fig. 3 Individual and mean plasma to whole blood concentration ratios over time for the six dogs with rapid plasma separation. *Error bars* indicate the standard error of the mean. *Solid line* is the curve fit to mean ratio data

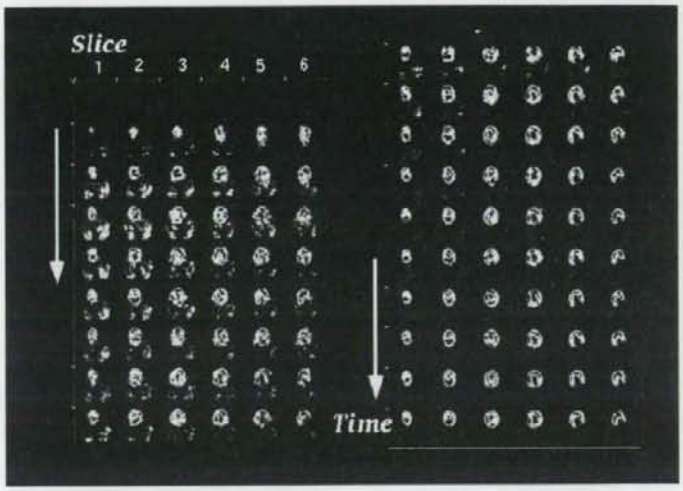

Fig. 4 A typical example of sequential SPECT images of the myocardium for six representative slices after intravenous injection of ^{201}Tl into a canine at rest

Fig. 3. The plasma to whole blood ratio curves could be approximated by the following equation:

$$R_{pl/wb} = A_0 e^{-\lambda(t+\Delta t)^2} + A_1\left(1 - e^{-\lambda_2(t+\Delta t)}\right), \quad (3)$$

which resulted in A_0=1.303±0.045, A_1=0.7649±0.0056, λ_1=0.03636±0.0039 min^{-1}, λ_2=0.1263±0.0077 min^{-1} and Δt=0.9516±0.41 min. The correlation coefficient for the fit was r=0.995.

Figure 4 shows a typical example of sequential images after the intravenous injection of ^{201}Tl for six representative slices of a dog studied at rest. It can be seen that ^{201}Tl appeared in the ventricular chambers first and then gradually accumulated homogeneously into the left myocardium. The quality of these images is reasonably good, indicating that our approach of estimating the kinetic parameters by NLLSF is feasible without excessive noise

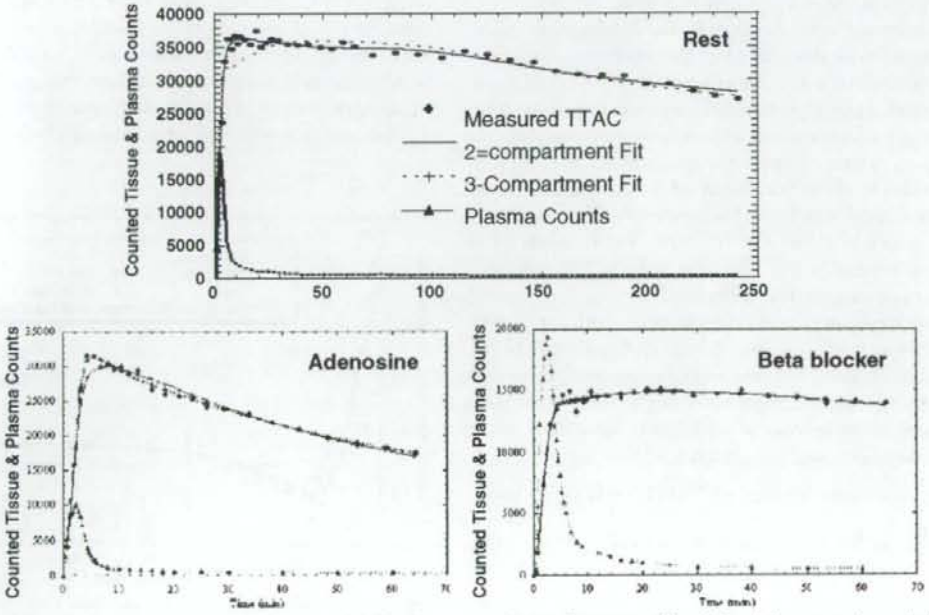

Fig. 5 TTACs and two- and three-compartment model fits for a resting, adenosine (increased MBF) and beta-blocker (reduced MBF) study. Note the different time scales for the resting study because resting studies were collected for 4 h compared to ≈1 h for the pharmacological intervention studies