

Basic Study on Atherosclerosis Imaging : The Effect of Foam-cell Formation on *S*-[methyl-¹⁴C]-D/L-methionine Uptake by THP-1-derived Macrophages

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Abstract

Objective: Monocyte-derived macrophage accumulation and subsequent foam-cell formation are large contributors to atherosclerotic plaque formation. In the present study, we studied the uptake of the optical isomers of *S*-[methyl-¹⁴C]-D/L-methionine (D/L-[¹⁴C]-Met) during differentiation of human-derived THP-1 monocytes to macrophage foam cells using positron emission tomography (PET) with D/L-[¹⁴C]-Met as a qualitative diagnostic agent to assess plaque vulnerability. We also investigated whether it is possible to augment D/L-[¹⁴C]-Met accumulation in THP-1-derived macrophages by stimulating an uptake-associated amino acid exchange transporter.

Methods: THP-1 cells (1×10⁶ cells/ml) were cultured in 35-mm-diameter dishes with 1 ml of Roswell Park Memorial Institute medium containing fetal bovine serum and L-glutamine for 2 days. Phorbol 12-myristate 13-acetate (PMA) was added to each dish at a concentration of 100 ng/ml and cells were cultured for 6, 12, 24, 48, and 72 hours to induce differentiation into macrophages. Cells were cultured for 72 hours following PMA treatment to allow for differentiation and then treated with acetylated low-density lipoprotein (Ac-LDL, 50 μg/ml) and cultured for 6, 12, 24, 48, and 72 hours to induce macrophage foam-cell formation. Cells were stained with oil Red O at different time points after PMA and Ac-LDL treatment, and the 10-min radioactivity uptake of D/L-[¹⁴C]-Met (3.7 kBq) was measured. Cells were also treated with L-tyrosine ethyl ester (L-Tyr-OEt) to stimulate exchange transport, and the 60-min uptake of D/L-[¹⁴C]-Met (3.7 kBq) was compared with untreated cells.

Results: During PMA-induced macrophage differentiation, D/L-[¹⁴C]-Met uptake increased rapidly, reaching a maximum at 48 hours, with approximately 5.8% of the treatment dose in the L-form and approximately 2.2% in the D-form. Uptake of both forms decreased thereafter. During foam-cell formation after Ac-LDL treatment, uptake decreased rapidly in the first 12 h and continued decreasing up to 72 h. Uptake stimulation by L-Tyr-OEt resulted in an approximately 3-fold increase in D/L-[¹⁴C]-Met uptake compared with the respective control treatments that did not contain L-Tyr-OEt (*p*<0.00005).

Conclusion: D/L-[¹⁴C]-Met was observed to accumulate in THP-1-derived macrophages, and this accumulation was enhanced by L-Tyr-OEt in this study. In vivo application of this system in future PET basic studies using D/L-[¹⁴C]-Met could facilitate clearer atherosclerosis imaging. Comparisons with other labeled compounds using animal models are anticipated.

Key words: atherosclerosis imaging, THP-1 cells, differentiation, macrophages, *S*-[methyl-¹⁴C]-D/L-methionine

1. Introduction

In recent years, atherosclerosis has become recognized as a chronic inflammatory disease in which inflammation is known to be involved in both the onset and development of the lesion, with eventual plaque rupture. Plaques that are prone to rupture are termed “vulnerable” and are pathologically characterized by a lipid-rich core, a thin fibrous cap that covers the area, and infiltration by inflammatory cells such as lymphocytes and macrophages.¹⁾ Macrophage accumulation following vascular endothelial damage is the most significant histopathological finding of an atherosclerotic plaque and is considered to be profoundly involved not only in plaque formation but also in plaque destabilization. In the lipid core of the plaque, macrophages take up low-density lipoproteins (LDLs) such as oxidized LDL and acetylated LDL via scavenger receptors and subsequently become foam cells in which cholesterol esters accumulate. An increased number of foam cells in the intima of the blood vessels promotes intimal thickening and plaque formation, and this induces the formation of a necrotic core due to the continuous death of foam cells, leading to weakening of the atherosclerotic plaque.²⁾

Some researchers have reported that fluorine-18 fluorodeoxyglucose (^{18}F -FDG) positron emission tomography (PET) is a promising tool for detecting vulnerable atherosclerotic plaques, depending on the extent of macrophage infiltration into the atherosclerotic lesion.³⁻⁵⁾ In evaluating glucose derivatives for PET analyses of macrophages and foam cells in atherosclerotic lesions, Ogawa et al. reported that ^{18}F -FDG enables visualization of the early stage of foam-cell formation in vulnerable plaques. Changes in hexokinase activity tended to accompany changes in ^{18}F -FDG uptake.^{6,7)}

In the present study, we examined the accumulation of amino acid PET agents in macrophages and foam cells of atherosclerotic lesions. Using the radiolabeled compounds ^{14}C -L-methionine and ^{14}C -D-methionine (D/L- ^{14}C -Met) as PET agents, we investigated the uptake of these ^{14}C -labeled amino acids during the differentiation process of THP-1 cells, which are human monocytes established in 1980 from a 1-year-old child

with acute monocytic leukemia. One of the primary characteristics of this cell line is that the cells differentiate and mature into macrophages following stimulation with phorbol 12-myristate 13-acetate (PMA).⁸⁾

The transporter in system L, which transports D/L- ^{14}C -Met, is a Na^+ -independent exchanger that transports neutral amino acids with large, neutral side chains, including tyrosine, by exchanging one extracellular amino acid with one intracellular amino acid. Based on our previous study,⁹⁾ we hypothesized that the ethyl esters of neutral amino acids such as tyrosine pass through the lipid bilayer of the cell membrane due to their fat-soluble nature, undergo enzymatic hydrolysis in the cell, and are then converted to neutral amino acids. These neutral amino acids then serve as substrates for intracellular system L exchange transport, resulting in the augmentation of amino acid uptake. Based on this principle, we investigated the augmented accumulation of D/L- ^{14}C -Met in THP-1-derived macrophages.

2. Materials and methods

2.1. Materials

D/L- ^{14}C -Met was purchased from American Radiolabeled Chemicals Inc. THP-1 (human acute monocytic leukemia, JCRB0112) cells were purchased from JCRB. Plastic tissue culture dishes (diameter 35 mm, Cat. no. 3000-035) were purchased from IWAKI. Other chemicals of reagent grade were purchased from Funakoshi or Kanto Chemical Co., Inc.

2.2. Maintenance of THP-1 cells in culture

THP-1 cells were plated onto 35-mm diameter dishes in 1 ml of Roswell Park Memorial Institute (RPMI) medium containing fetal bovine serum (FBS) and L-glutamine at a density of 1×10^5 cells/ml and cultured at 37°C , pH 7.4, and 5% CO_2 for 6 days. Cells were counted every 24 h.

2.3. Induction of THP-1 cell differentiation

Using the culture system described in section 2.2 above, THP-1 cells were plated at a density of 1×10^6 cells/ml and cultured at 37°C , pH 7.4, and 5% CO_2 for 2 days. PMA

(100 ng/ml) was added to the dishes, and the cells were cultured for 6, 12, 24, 48, and 72 h to induce differentiation into macrophages. Cells were cultured for 72 h after PMA treatment to ensure sufficient differentiation into macrophages and then treated with acetylated low-density lipoprotein (Ac-LDL; 50 µg/ml) and cultured for 6, 12, 24, 48, and 72 h to induce macrophage foam-cell formation.

To confirm the progression of macrophage foam-cell formation, oil red O lipid staining was performed as described below. First, 30 mg of oil red O powder was dissolved in 10 ml of 100% isopropyl alcohol (2-propanol) and mixed well to make a stock solution. To prepare a working solution, 3 ml of the stock solution and 2 ml of distilled water (3:2 ratio) were mixed well, left to stand for 10 min, and subsequently filtered. After removing 1 ml of medium from the dish, the cells were washed twice with 1 ml of phosphate-buffered saline (PBS; NaCl, 137 mM; KCl, 2.7 mM; Na₂HPO₄•12H₂O, 8 mM; KH₂PO₄, 1.5 mM; CaCl₂•2H₂O, 1.8 mM; MgCl₂•6H₂O, 1.0 mM; 37°C; pH 7.4) and fixed with 1 ml of 10% formalin for 10 min. After removing the formalin, the cells were washed again twice with 1 ml of PBS and incubated with 1 ml of 60% isopropanol for 1 min. After removing the isopropanol, filtered oil red O working solution was added and incubated for 15 min for staining. Oil red O working solution was removed after staining was completed, and the cells were washed once with 1 ml of 60% isopropanol and twice with 1 ml of PBS. The cells were then examined under a microscope and imaged using a digital microscope camera.

2.4. Time course of D/L-[¹⁴C]-Met uptake

The uptake of both [¹⁴C]-Met optical isomers was measured at different time points in macrophages derived from THP-1 cells following a 48-h PMA treatment. After removing 1 ml of culture medium from the dish, 1 ml of PBS was added and the cells were incubated for 10 min. After removing the PBS, the cells were incubated with 1 ml of PBS containing 3.7 kBq of D/L-[¹⁴C]-Met for 5, 15, 30, and 60 min. After removing the PBS, the surface of the cell layer was washed twice with 1 ml of PBS (4°C) and subsequently incubated with 1 ml of NaOH for 1 day to lyse the cells. Radioactivity was measured in 200 µl

of each culture sample using a liquid scintillation counter (PerkinElmer Inc., Tri-Carb 2910TR).

2.5. Uptake of D/L-[¹⁴C]-Met by THP-1 cells during differentiation and foam-cell formation

The uptake of both D/L-[¹⁴C]-Met optical isomers was evaluated in each plot. After removing 1 ml of the culture medium from the dish, the cells were incubated in 1 ml of PBS for 10 min. After removing the PBS, the cells were incubated with 1 ml of PBS containing 18.5 kBq of D/L-[¹⁴C]-Met for 10 min. After removing the PBS, the surface of the cell layer was washed twice with 1 ml of PBS (4°C) and incubated with 1 ml of NaOH for 1 day to lyse the cells. Radioactivity was measured in 200 µl of each culture sample using a liquid scintillation counter (PerkinElmer Inc., Tri-Carb 2910TR).

2.6. Uptake augmentation study using an amino acid ethyl ester

The augmentation of uptake stimulated by an amino acid ethyl ester exchange transport mechanism that utilizes the system L exchange transporter was investigated at 24 h after PMA addition to the dishes, as described above. After removing 1 ml of the culture medium from the dish, the cells were incubated in 1 ml of PBS (37°C, pH 7.4) for 10 min. After removing the PBS, the cells were incubated in 1 ml of PBS containing 3.7 kBq of D/L-[¹⁴C]-Met and 1 mM L-tyrosine ethyl ester (L-Tyr-OEt) for 60 min. After removing the PBS, the surface of the cell layer was washed twice with 1 ml of PBS (4°C) and incubated in 1 ml of NaOH for 1 day to lyse the cells. Radioactivity was measured in 200 µl of each culture sample using a liquid scintillation counter.

2.7. Statistical analysis

Data were collated as the mean ± standard deviation of five measurements, and each experiment was performed in duplicate. Results were analyzed using Student's t test; p-values <0.01 were considered to statistically significant.

3. Results

3.1. Maintenance of THP-1 cells in culture

The growth curve of THP-1 cells is shown in Figure 1. Cell counts for plating and culture duration were determined from the growth curve.

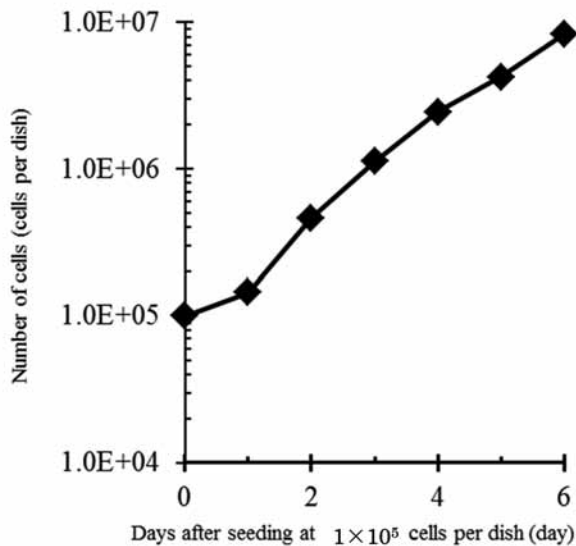


Figure 1. Growth curve of THP-1 cells.

3.2. Induction of THP-1 cell differentiation

Oil red O staining results are shown in Figure 2. At 12 h after Ac-LDL treatment, the cell count reached a maximum, and the greatest intensity in red-orange staining was observed in the dish as a whole. However, cells with extremely dark red-orange staining were observed after 12 h, even though the cell count had decreased. These cellular changes were consistent with those reported by Via *et al.*⁸⁾

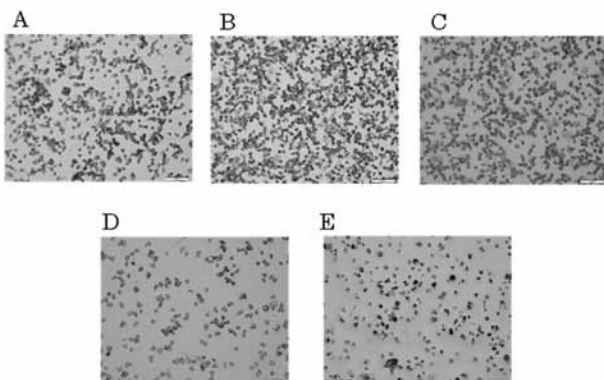


Figure 2. Oil red O staining of THP-1-derived macrophages at 6 (A), 12 (B), 24 (C), 48 (D), and 72 (E) h after PMA treatment.

3.3. Time course of D/L-[¹⁴C]-Met uptake

The time course of D/L-[¹⁴C]-Met uptake by macrophages derived from THP-1 cells following a 72-h PMA treatment is shown in Figure 3. At 15 min, uptake of the D-form was $3.6\% \pm 0.24(\%)$ and that of the L-form was $4.15 \pm 0.11(\%)$, and at 30 min, uptake of the D-form was $4.04 \pm 0.16(\%)$ and that of the L-form was $4.78 \pm 0.15(\%)$. A statistically significant difference in uptake between isomers was observed at both 15 and 30 min ($p < 0.02$, $p < 0.004$, respectively).

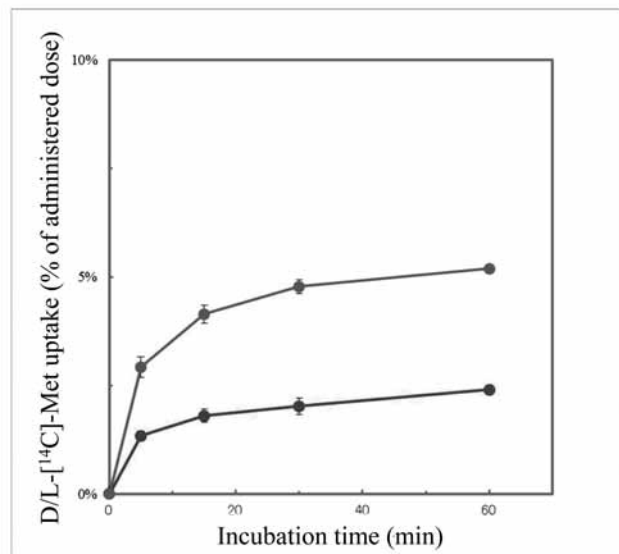


Figure 3. Time course of D/L-[¹⁴C]-Met uptake by THP-1-derived macrophages 72 h after PMA treatment. The upper line represents L-[¹⁴C]-Met uptake, and the lower line represents D-[¹⁴C]-Met uptake.

3.4. Uptake of D/L-[¹⁴C]-Met by THP-1 cells during differentiation and foam-cell formation

The uptake of D/L-[¹⁴C]-Met by THP-1 cells after PMA treatment is shown as a curve in Figure 4A. D/L-[¹⁴C]-Met uptake by macrophages after Ac-LDL treatment is shown as a curve in Figure 4B. As shown in Figure 4A, uptake increased rapidly after PMA treatment. Uptake of both the L- and D-forms decreased after 48 h. Figure 4B clearly shows that uptake continued to decrease after Ac-LDL treatment. In all conditions and time points examined in this study, uptake of the L-form of [¹⁴C]-Met was greater than that of the D-form.

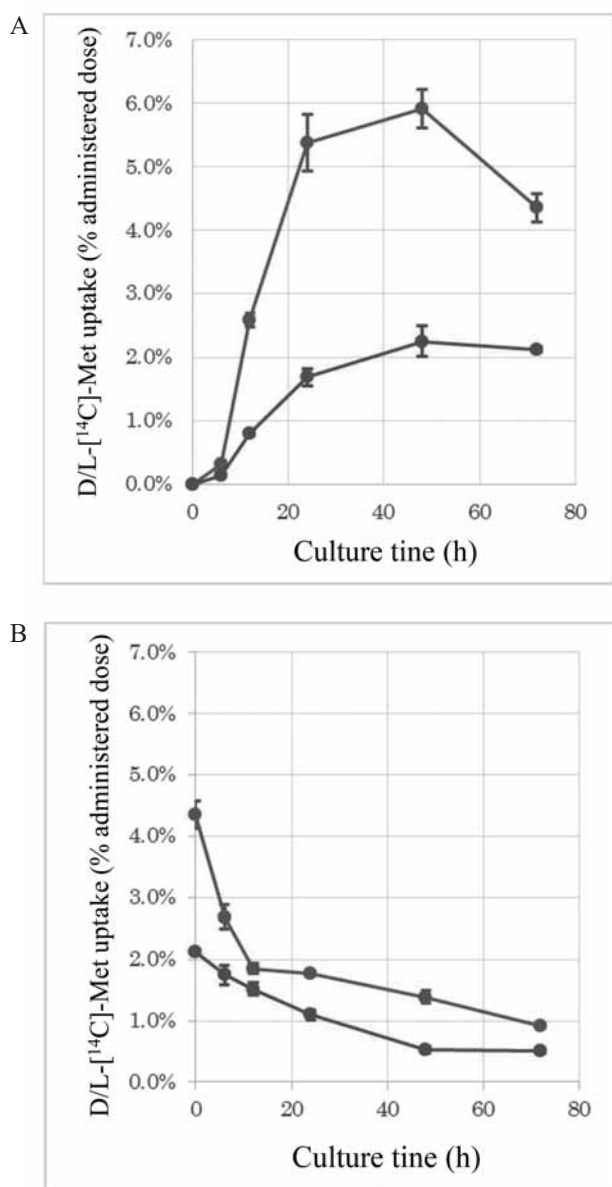


Figure 4. D/L-[¹⁴C]-Met uptake curve after PMA treatment (A), D/L-[¹⁴C]-Met uptake curve after Ac-LDL treatment (B). The upper line represents L-[¹⁴C]-Met uptake, and the under line represents D-[¹⁴C]-Met uptake. Cells were incubated for 10 min with D/L-[¹⁴C]-Met.

3.5. Uptake augmentation study using an amino acid ethyl ester

Figure 5 shows D-[¹⁴C]-Met and L-[¹⁴C]-Met uptake by control and L-Tyr-OEt-treated cells. The data are expressed as the ratio relative to each control. The D-[¹⁴C]-Met uptake ratio was 1.00 ± 0.28 in control cells and 3.31 ± 0.10 in L-Tyr-OEt-treated cells, and this difference was statistically significant ($p < 0.00005$). The L-[¹⁴C]-Met uptake ratio was 1.00 ± 0.03 in control cells (uptake level: $6.90\% \pm 0.18\%$) and 3.27 ± 0.20 in L-Tyr-

OEt-treated cells, and this difference was also statistically significant ($p < 0.00008$).

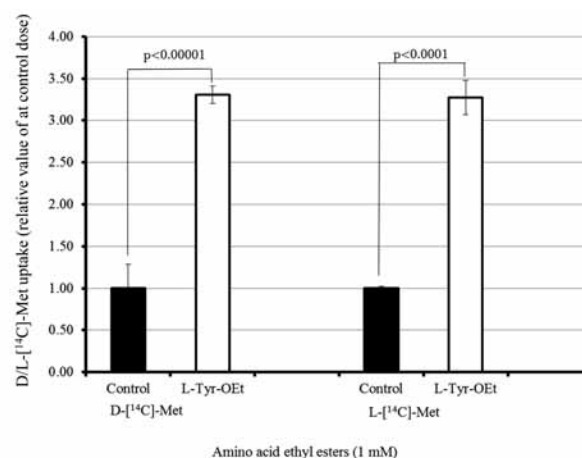


Figure 5. Uptake augmentation by amino acid ethyl esters (D/L-[¹⁴C]-Met uptake by macrophages treated simultaneously with L-Tyr-OEt is shown. Values are expressed as a ratio relative to each control 1.0).

4. Discussion

Diseases such as myocardial infarction and cerebral infarction are caused by atherosclerosis, a pathologic condition that can occur systemically. These diseases are a critical issue in our aging society. The quality of the plaque is the greatest concern when atherosclerosis is diagnosed, and determining the location and degree of vulnerable plaques (which have a greater risk of rupturing than stable plaques) is critical. Nuclear medicine tests provide specific and non-invasive in vivo visualization data at the molecular and cellular levels through the administration of radioisotope-labeled probes. These tests are used for the qualitative diagnosis of atherosclerotic plaques. In addition, single-photon emission computed tomography (SPECT) and especially PET allow for quantitative imaging of factors that facilitate determinations of plaque vulnerability based on morphologic information as well as cellular-level and gross biological changes in the lesion area.

Risk factors of atherosclerosis include hypertension, hyperlipidemia, and diabetes. When vascular endothelial damage and intimal swelling caused by these conditions trigger an inflammatory response, monocytes infiltrate through the vascular wall and differentiate into macrophages.

In hyperlipidemia, excess LDL particles infiltrate through the vascular wall and undergo oxidative modification, leading to endothelial cell damage. Macrophages that become activated exacerbate the inflammation while proliferating, take up these oxidized LDL particles, and eventually become foam cells. Atheromatous plaques are generated through such a process and contribute to the progression of atherosclerosis. Myocardial infarction and cerebral infarction are caused by a decrease in blood flow caused by a narrowing of the blood vessel caliber due to increased atheromatous plaques as well as the generation of thrombi due to plaque rupture.^{10, 11)}

The THP-1 cells used in the present study were from a human monocytic cell line derived from acute monocytic leukemia. THP-1 cells differentiate and mature into macrophages following PMA stimulation.⁸⁾ As shown in Figure 1, these monocytic THP-1 cells proliferated normally in the culture system used in this study.

During the induction of macrophage differentiation, as shown in Figure 2, red-orange staining was observed at the highest intensity in macrophages at 12 h after Ac-LDL treatment. Up to this time point, there was a trend toward macrophage proliferation. However, at 12 h and later, macrophage foam cells detached from the surface of the dish, leading to a decrease in the cell count. Nonetheless, as shown in Figure 4A, D/L-[¹⁴C]-Met uptake continued to increase up to 48 h. This was postulated to be because the monocytes differentiated into macrophages, which then became activated, and this consequently led to the upregulation in the amino acid transporter that is responsible for D/L-[¹⁴C]-Met uptake. As a result, greater uptake was observed. In the present experimental system, it appears that expression of the amino acid transporter responsible for D/L-[¹⁴C]-Met uptake by macrophages peaked around 48 h after PMA treatment.

Activated macrophages can be classified based on function, and at least two types have been identified to date. M1 macrophages are immune-responsive and exhibit antibacterial and antiviral activities. In contrast, M2 macrophages are immunosuppressive and are responsible for tissue repair and angiogenesis.¹⁾ Since atherosclerosis is an inflammatory disease triggered by an immune response, the involvement of M1 macrophages

would be expected to play a pathologic role. However, recent studies have indicated three new classifications of macrophage activation (*e.g.*, a host defense system, wound healing system, and immunoregulatory system), and there is ongoing discussion regarding the multifaceted and varied properties of macrophage activation.^{1, 2)} Based on the above, even if activated macrophages can be captured as an image, further analysis would still be necessary in order to determine the type of macrophage and more specifically, to determine whether or not the accumulation of such activated macrophages is involved in atherosclerotic plaque formation.

As shown in Figure 4B, uptake decreased rapidly after Ac-LDL treatment. Since obvious morphological changes in the cells and a decrease in radioactivity uptake were observed after Ac-LDL treatment, it is likely that there was a progression in foam-cell formation. We hypothesize that this had occurred because the expression or function of the neutral amino acid transporter attenuated progression of foam-cell formation.

Previously identified pathways for the uptake of neutral amino acids by cells include transport systems L, A, and ASC. Systems A and ASC are Na⁺ dependent and can be distinguished by the pH-dependence of the system and specific blockade by non-natural amino acids. System A is Na⁺ dependent, and uptake can be blocked with α -(methylamino)-isobutyric acid (MeAIB). System ASC is also Na⁺ dependent, but blockade by MeAIB is not observed in this system. System L is Na⁺ independent and is blocked specifically by the non-natural amino acid 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid.¹²⁻¹⁴⁾ In our experimental system, it was revealed that the majority of D/L-[¹⁴C]-Met uptake occurs through system L (data not shown). Regarding [¹⁴C]-Met, a large difference in uptake between the L-form and its optical isomer D-form has been reported.¹⁵⁻¹⁷⁾ Even during THP-1 differentiation, [¹⁴C]-L-Met uptake in the present study was 2- to 3-fold greater than that of [¹⁴C]-D-Met (Figure 3).

As shown in Figure 5, uptake of both the D- and L- forms of [¹⁴C]-Met increased with the simultaneous administration of L-Tyr-OEt. In accordance with the expected mechanism shown in Figure 6, it appears that our observation was a result of L-Tyr-OEt-induced

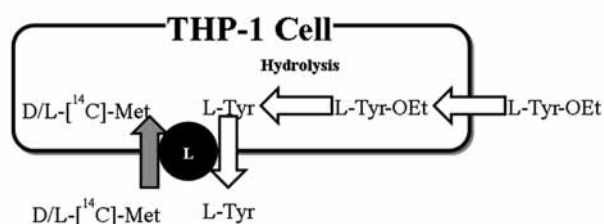


Figure 6. Postulated mechanism of uptake augmentation through ethyl ester exchange transport.

stimulation of system L exchange transport.^{9,18)} With the development of a suitable method to deliver L-Tyr-OEt to atherosclerotic sites, an improvement in the sensitivity of detecting plaques at the lesion site and a reduction in the dose of radioactive drugs needed would be anticipated. In addition, because the D-form is cleared from the blood circulation faster than the L-form,¹⁹⁾ D-[¹⁴C]-Met may provide images with better diagnostic accuracy if a comparable level of uptake can be expected, considering the contrast between the blood and plaque, which is a key component of lesions in blood vessel walls.

In the future, basic PET studies using the D-isomer of [¹¹C]-Met in particular should provide clearer atherosclerosis imaging with higher contrast and in less time. Subsequent comparisons with other labeled compounds, such as FDG, using animal models is anticipated for eventual in vivo application of this system.

Conflict of interest

The authors declare that they have no conflict of interest.

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和文抄録

単球由来マクロファージ集積とその後の泡沫化は、アテローム性動脈硬化性病変の形成早期における中心的病態である。ヒト由来 THP-1 単球のマクロファージ泡沫細胞化過程での S-[methyl- ^{14}C]-D/L-methionine (D/L-[^{14}C]-Met) の各光学異性体の取り込み、プラークの脆弱性評価目的の定性的診断薬としての S-[methyl- ^{11}C]-D/L-methionine (D/L-[^{11}C]-Met) 集積、およびアミノ酸交換輸送体を刺激することによって、THP-1 由来マクロファージにおける D/L-[^{14}C]-Met 集積増大が可能かどうかを調べた。細胞を L-チロシンエチルエステルで処理して交換輸送を刺激し、D/L-[^{14}C]-Met の 60 分間の取り込みを未処理細胞と比較した。D/L-[^{14}C]-Met は THP-1 由来のマクロファージに集積し、それは L-Tyr-OEt を用いると増加した。今後の課題として、*in vivo* モデル動物実験で positron emission tomography (PET) による画像的評価を行い、臨床で用いられている fluorine-18 fluorodeoxyglucose (^{18}F -FDG) や他のアミノ酸標識化合物との比較・検討を基礎的に行うことが期待される。

キーワード: 動脈硬化イメージング, THP-1 細胞, 分化, マクロファージ, S-[methyl- ^{14}C]-D/L-methionine