厚生労働科学研究費補助金

エイズ対策研究事業

HIV陽性者における進行性多巣性白質脳症に対する高精度検査技術の開発および診断への応用

(H24-エイズ-若手-002)

平成２６年度 総括研究報告書

平成２４年度～平成２６年度 総合研究報告書

研究代表者　中道　一生

(国立感染症研究所)

平成２７(２０１５)年５月

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※ 若手育成型課題において単独で研究を実施したため分担研究報告は該当しない。

Ⅰ．平成２６年度 総括研究報告書

１）総括研究報告

（若手育成型課題において単独で実施したため分担研究報告は該当しない）

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研究代表者　中道 一生
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研究要旨

進行性多巣性白質脳症(Progressive Multifocal Leukoencephalopathy, PML)は、JCウイルス(JCV)に起因する脱髄疾患であり、HIV陽性者を中心とした免疫不全患者等において発生する。PMLの診断では、脳脊髄液を用いたJCVゲノムDNAのリアルタイムPCR検査が主流となっている。この検査手法は極めて鋭敏であるが、病原性のない持続感染型JCVの混入、もしくは検体間の汚染によって偽陽性を生じる危険性を有している。PMLが疑われる患者では基礎疾患の治療において化学療法や免疫抑制療法を受けていることが多く、偽陽性はPMLの診断のみならず基礎疾患の治療方針にまで悪影響を及ぼしかねない。PMLを引き起こすタイプのJCVは、ウイルスゲノムの調節領域に多様な変異を有している。そこで、脳脊髄液中に存在するJCVの変異パターンを迅速に識別することで、変異ウイルスの有無およびJCVのPCR検査における偽陽性の可能性を解析するための検査技術の開発を着想した。本研究は、「高解像度融解曲線分析(High-Resolution Melting analysis, HRM)法を用いて、JCVのゲノムDNAに生じるランダムな変異を迅速に識別するための検査系を確立し、PMLの高精度診断技術へと応用すること」を目的とする。前年度までの本研究では、JCVゲノムの変異領域を標的としたHRM検出系を開発し、臨床面でのバリデーションを経てPMLの診断技術として実用化することに成功した。研究最終年度となる今年度では、PMLの治療において経時的に採取された脳脊髄液、もしくは生検等において採取された脳組織におけるJCVの変異パターンをHRMによって解析することで本検査技術の応用性を評価した。HRM検査系を用いた場合、脳脊髄液中のJCVの変異パターンは数ヶ月単位のフォローアップにおいても安定しており、複数の陽性検体を扱う場合においても患者由来のウイルスを照合することができた。また、HRMスキャニングは、変異型JCVのクローニングおよびスクリーニングにおいても有用なツールであることを示した。これらの結果から、HRMによるJCVの変異スキャニングは、PMLの診断だけでなく、フォローアップ検査や脳組織検査、さらにはPMLの病態解明における基礎的研究においても有用であることを示した。

研究分担者
該当なし。（若手育成型の研究課題であり、研究代表者が単独で実施した）

A.研究目的

進行性多巣性白質脳症(Progressive Multifocal Leukoencephalopathy, PML)は、ポリオーマウイルス科のJCウイルス(JCV)に起因する脱髄疾患であり、患者の約30～50%をHIV陽性者が占める。JCVは多くの成人に持続感染しており、免疫抑制に伴って変異型ウイルスが出現し、大脳白質等を破壊する。治療がなされない場合、ほぼ全てのPML患者が発症から1年以内に死に至る。AIDS患者の場合にはHIV陽性であることが判明する以前の段階でPMLを発症するケースも珍しくない。

PMLの診断では脳脊髄液を用いたJCVゲノムDNAのPCR検査が有効である。また、近年では高感度な定量的リアルタイムPCRを用いた検査系が数多く開発され、それらはPMLの診断における一般的な検査手法として活用されている。しかし、この手法は健常人においても持続感染している病原性のないアーキタイプJCVの混入、もしくは陽性検体から陰性検体への汚染によって偽陽性を生じるリスクを有している。研究代表者らは、より確実なPMLの診断や治療を目的として偽陽性の有無を迅速に調べるための検査系が必要であると考えた。

PMLを引き起こすタイプのJCVは、ウイルスゲノムの調節領域に多様な変異を有している。そこで、脳脊髄液中に存在するJCVの変異パターンを迅速に識別することで、変異ウイルスの有無およびJCVのPCR検査における偽陽性の可能性を解析するための検査技術の開発を着想した。本研究は、「高解像度融解曲線分析(High-Resolution Melting analysis, HRM)法を用いて、JCVのゲノムDNAに生じるランダムな変異を迅速に識別するための検査系を確立し、PMLの高精度診断技術へと応用すること」を目的とする。

前年度までの研究では、JCVゲノムの変異領域を標的としたHRM検査系を確立した後、臨床面でのバリデーションを実施し、本検査系をPMLの診断技術として実用化することに成功した。平成26年度(最終年度)では、PMLの治療において経時的に採取された脳脊髄液、もしくは生検等において採取された脳組織におけるJCVの変異パターンをHRMによって解析することで本検査技術の応用性を評価した。

B.研究方法

健常人に持続感染しているJCVのゲノム配列は安定しており、宿主の終生を通じて大きな変異は認められない。一方、PMLを引き起こすJCVではゲノムの転写調節領域(以下、調節領域)において患者個人レベルの多様な変異が生じる。本検査系は、リアルタイムPCR法によって調節領域を増幅した後、そのDNA断片の配列の相違(解離温度の差異)をHRMによって測定することを原理としている。これらの解析には、前年度と同様の機器および試薬等を用いた。

1）PMLの治療におけるHRM検査系の有用性の評価

PMLの診断後にフォローアップ検査が実施された患者26名の脳脊髄液を研究の対象とした。患者らは基礎疾患としてAIDSもしくは血液疾患、自己免疫疾患等を有しており、全ての検体の脳脊髄検査を研究代表者が担当した。

PML診断時および1～3ヶ月後に採取された脳脊髄液(26組、計52検体)からDNAを抽出し、JCV.ゲノムの調節領域をリアルタイムPCRによって増幅した。PCRに引き続き同一のチューブ内においてHRMを実施した後、調節領域の解離温度を比較することで、「PMLの治療中に実施されるフォローアップ検査においてもJCVの変異パターンを患者個人レベルで照合しうるか否か」を解析した。

２）脳組織検査および基礎研究におけるHRM検出系の応用

本研究は国立感染症研究所感染病理部および他の医療機関との共同研究として実施した。病理学的検査を目的として提出されたPML患者の脳組織サンプルを研究に用いた。HIV陽性者を含む12名のPML患者の脳組織DNA(複数部位を含む18検体、うち生検10検体)におけるJCVの変異パターンをリアルタイムPCR-HRMによって解析することで、「脳組織検体を患者個人レベルで識別しうるか否か」を解析した。また、調節領域をプラスミドに接続し、大腸菌に導入することで変異型JCVのDNAライブラリーを作製した。それぞれの大腸菌の一部を熱処理した後、各配列の変異パターンをHRMによって比較することで、「JCV-DNAクローンのハイスループットスクリーニングに応用できるか否か」を解析した。

（倫理面への配慮）

本研究は、国立感染症研究所ヒトを対象とする医学研究倫理審査委員会の承認を受けた後、適切な配慮のもとに実施された。

C.研究結果

１）PMLの治療におけるHRM検査系の有用性の評価

PMLと診断された患者では、抗レトロウイルス療法もしくは免疫抑制の解除等が行われ、脳脊髄液JCV量の変動をモニターすることが多い。PMLの診断時および治療中のフォローアップにおいて採取された脳脊髄液検体を対象として、それらの検体に含まれるJCVの変異パターンがHRMによって患者レベルで識別しうるか否かを解析した。26名のPML患者から採取された脳脊髄液検体では、その全てにおいて変異型JCVが検出された。また、各々の検体中のJCVは患者個人レベルで異なった変異パターンを示した。PMLの診断時の初回検査、およびフォローアップ検査において検出されたJCVの変異を同一患者において比較したところ、28組中26組においてHRMにおける変異パターンが一致した。他の2組においては、変異パターンが若干変化したものの、解離温度のピークは同様であった。これらの結果から、HRM検査系を用いた場合、脳脊髄液中のJCVの変異パターンは数ヶ月単位のフォローアップにおいても安定しており、長期に亘って患者由来のJCVを照合しうることが分かった。

２）脳組織検査および基礎研究におけるHRM検出系の応用

PML患者の脳組織から抽出されたDNAを鋳型として、HRMによる調節領域の解離温度を比較したところ、それぞれの検体に含まれるJCVの変異パターンは、患者レベルで異なることが分かった。また、各々の検体におけるJCVの調節領域をプラスミドにクローニングすることで、約1,700クローンを含む変異型JCVのDNAライブラリーを作製した。加えて、各クローンを有する大腸菌からの核酸抽出を省略し、菌体浮遊液をHRMの鋳型として直接的に用いるための条件を至適化した。この系を用いることで、変異型JCVのDNAクローンをハイスループットで選別することが可能となった。上記の結果より、HRM検出系は、PML患者における

JCVの変異を解析する上でも有用なツールとなりうることが分かった。

D.考察

リアルタイムPCRはウイルス検査における汎用技術となっており、優れた迅速性、感度、特異性を有する。一方、本法の最大のリスクである偽陽性については未だ検討の余地があり、汚染の可能性を迅速に調べるためのpost-hoc検査系の開発が十分になされていない。PCR検査において最も偽陽性を引き起こす可能性が高い事象は、陽性対照DNAによるサンプル汚染である。多くのPCR検査系では、このリスクに対して陽性対照DNAの断片長や制限酵素切断部位、プローブ結合部位等を改変するといった対策が講じられている。

しかし、リアルタイムPCR検査ではウイルスゲノムにおいて高度に保存された領域を標的配列として使用することが多い。そのため、複数の陽性検体が認められた際に、PCRの増幅産物の配列の解析によって陽性検体から陰性検体への汚染であるか否かを迅速に判定することは困難である。そのようなケースでは、検査環境のクリーンアップを行った後、当該サンプルについて、再度の核酸抽出もしくは検体採取といった非効率的な対応に迫られることがある。

PMLを引き起こすJCVは、ウイルスゲノムの調節領域に患者個人レベルの多様な変異を生じるというユニークな性質を有している。この点に着目し、ルーチンのPCR検査において陽性を示した場合の確認検査として、転写調節領域の配列を解析するというアプローチが古くから実施されている。しかし、PML患者の脳脊髄液には、複数のJCV変異体が含まれており、ダイレクトシーケンシングによる塩基配列の決定が困難となるケースが多い。そのため、PCR検査において偽陽性が疑われた場合には、ウイルスゲノムの転写調節領域をプラスミドにクローニングした後、多数のDNAクローンの塩基配列を決定し、複雑に変異した配列をin silicoにて解析する必要がある。これら一連の工程には、煩雑かつ長期間の作業、および大きな経済的コストを要するためルーチン検査での実施は困難である。

前年度までの研究では、PML型JCVのゲノムにおける多様な変異パターンをスキャンするためのHRM検査系を確立し、PML患者の脳脊髄液を患者個人レベルで識別することに成功した。また、多数の臨床検体を用いたバリデーションを経て本検査系の実用化に成功した。本検査系を用いることで、ウイルスゲノムのクローニングや次世代シーケンシング等を行うことなく、迅速かつ低コストにJCVの変異を解析することが可能となった。

今年度の研究では、HRM検出系の応用性を評価した。PMLに対する根本的治療法は確立されていないが、抗レトロウイルス療法もしくは免疫抑制を解除する等の治療によってPMLの進行が停止し、症状が改善する場合がある。そのため、治療前後における脳脊髄液中のJCVレベルを比較するケースが多い。本研究の結果、HRM検査系を用いることで、PMLの治療中に採取された検体であっても、患者レベルの変異パターンを測定することができた。フォローアップ検査を実施する際に本検査系を用いることで、シーケンシングを行うことなく、過去の検体との照合を行うことが可能となった。

また、本検出系は、脳組織中のヒトゲノムDNAによる影響を受けることなくJCVの変異パターンを識別したことから、脳組織を用いたJCV検査においても有用であることが示唆された。JCVの病原性には調節領域の変異が関与していることが示唆されており、本HRM検出系は、変異型ウイルスの解析においても有用なツールとなりうる。

E.結論

PMLを引き起こすJCVのゲノムでは患者個人レベルの多様な変異が生じる。検体中のJCVの変異パターンをHRMによって迅速にスキャンすることで、陽性検体を患者個人レベルで識別するための検査技術を開発した。また、HRMによるJCVの変異スキャニングは、PMLの診断だけでなく、フォローアップ検査や脳組織検査、さらにはPMLの病態機序に関する基礎的研究においても有用なツールとなりうることを示した。

F.健康危険情報

該当なし

G.研究発表

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H. 知的財産権の出願・登録状況

該当なし。

２）研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
該当なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nukuzuma, S, Nakamichi, K, Kameoka, M, Sugiura, S, Nukuzuma, C, Tasaki, T, Takegami, T.	TNF-α stimulates replication of JC virus efficiently in neuroblastoma cells.	J. Med. Virol.	86(12)	2026-2032	2014
Nakamichi K, Tajima S, Lim CK, Saijo M.	High-resolution melting analysis for mutation scanning in the non-coding control region of JC polyomavirus from patients with progressive multifocal leukoencephalopathy.	Arch. Virol.	159(7)	1687-1696	2014
Ohara H, Kataoka H, Nakamichi K, Saijo M, Ueno S.	Favorable outcome after withdrawal of immunosuppressant therapy in progressive multifocal leukoencephalopathy after renal transplantation: case report and literature review.	J. Neurol. Sci.	341(1-2)	144-146	2014
Nakamichi K, Lim CK, Saijo M.	Stability of JC virus DNA in cerebrospinal fluid specimens preserved with guanidine lysis buffer for quantitative PCR testing.	Jpn. J. Infect. Dis.	67(4)	307-310	2014
Shirai S, Yabe I, Kano T, Shimizu Y, Sasamori T, Sato K, Hirotani M, Nonaka T, Takahashi I, Matsushima M, Minami N, Nakamichi K, Saijo M, Hatanaka KC, Shiga T, Tanaka S, Sasaki H.	Usefulness of ^{11}C-methionine-positron emission tomography for the diagnosis of progressive multifocal leukoencephalopathy.	J. Neurol.	261(12)	2314-2318	2014

３） 研究成果の刊行物・別刷

Journal of Medical Virology 86:2026–2032 (2014)

TNF-α Stimulates Efficient JC Virus Replication in Neuroblastoma Cells

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JC polyomavirus (JCV) causes progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS) in immunocompromised patients, and particularly in the severe immunosuppression associated with acquired immunodeficiency syndrome (AIDS). HIV-1 can lead to the production of tumor necrosis factor-alpha (TNF-α) in the CNS. Our aim was to examine the effects of TNF-α on JCV gene expression and replication using a human neuroblastoma cell line, IMR-32, transfected with JCV DNA, M1-IMRb. Quantitative RT-PCR analysis of JCV large T antigen and VP1 mRNA, the viral DNA replication assay, and the DNase protection assay were carried out. TNF-α treatment of IMR-32 cells transfected with JCV DNA induced large T antigen mRNA and JCV DNA replication, while other effects on VP1 mRNA expression and virus production were marginal. In addition, ELISA analysis of the nuclear p65 subunit of nuclear factor κB (NF-κB), which is a hallmark of NF-κB pathway activation, of IMR-32 cells upon TNF-α treatment showed that TNF-α treatment activated the NF-κB pathway in IMR-32 cells. Taken together, our results suggest that TNF-α stimulation could induce JCV replication associated with the induction of JCV large T antigen mRNA through the NF-κB pathway in IMR-32 cells transfected with JCV DNA. Our findings may contribute to further understanding of the pathogenesis of AIDS-related PML. ***J. Med. Virol. 86:2026–2032, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: TNF-α; T antigen expression; IMR-32; NF-κB; DNA replication

INTRODUCTION

JC polyomavirus (JCV) causes progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (CNS) in immunocompromised patients [Major et al., 1992]. Demyelination in the brain of PML patients is caused by the destruction of oligodendrocytes, the myelin-producing cells of the CNS [Major et al., 1992], which are preferentially infected by JCV in the human brain. The pathological features of PML are the presence of cytolytic oligodendrocytes with giant nuclei, and unusual astrocytes with hyperchromatic nuclei. However, a previous report also demonstrated the ability of JCV to infect neurons and macrophages in the CNS [Major et al., 1992]. JCV propagates in the CNS, in particular, leading to PML in patients with severe immunosuppression, such as that associated with acquired immunodeficiency syndrome (AIDS).

In general, JCV persists in a latent state, in which viral protein expression and replication are not detectable after primary infection [Hou and Major,

Grant sponsor: The Research Committee of Prion Disease and Slow Virus Infection, Ministry of Health, Labor and Welfare of Japan; Grant number: H22-Nanchi-Ippan-013.; Grant sponsor: Grant-in-Aid for the Research on HIV/AIDS, Ministry of Health, Labor and Welfare of Japan; Grant number: H24-AIDS-Wakate-002.; Grant sponsor: Grant for Project Research from the High-Tech Center of Kanazawa Medical University (partial support); Grant number: H2011-10.

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Accepted 18 December 2013

DOI 10.1002/jmv.23886
Published online 10 January 2014 in Wiley Online Library (wileyonlinelibrary.com).

2000; Khalili et al., 2006]. However, HIV-1 can lead to the production of tumor necrosis factor-alpha (TNF-α) in the CNS [Benveniste, 1994; Yeung et al., 1995; Kaul et al., 2005]. It has been indicated that TNF-α stimulates JCV transcription in both the early and late phases of infection via nuclear factor κB (NF-κB) in a human oligodendroglioma cell line [Wollebo et al., 2011]. NF-κB, a transcriptional factor, is induced by TNF-α. However, it has also been reported that TNF-α did not stimulate JCV transcription or multiplication in human fetal glial cells [Atwood et al., 1995]. Thus, it remains unclear whether TNF-α stimulates JCV transcription and replication. A few cell lines are susceptible to JCV propagation. Previous studies with the human neuroblastoma cell line IMR-32 revealed that JCV was produced by serial passage, because three JCV (designated M1-IMRa, M1-IMRb, and M1-IMRc) had altered regulatory regions that were involved in adaptation. After transfection, M1-IMRb showed the highest virus replication in IMR-32 cells among the three adapted clones [Yogo et al., 1993]. Thus, IMR-32 cells transfected with M1-IMRb are a useful tool for studying the role of TNF-α in JCV replication.

In this study, the effects of TNF-α on the expression of JCV large T and VP1 genes in neuroblastoma IMR-32 cells were analyzed using a real-time RT-PCR assay, and viral genome replication was analyzed using a DNA replication assay. Moreover, JCV production in IMR-32 cells transfected with M1-IMRb was determined using real-time PCR analysis combined with DNase treatment. In addition, the effect of TNF-α on nuclear translocation of the p65 subunit of NF-κB in IMR-32 cells was determined.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Cytokines

IMR-32 cells were obtained from DS-Pharma Biomedical (Osaka, Japan). IMR-32 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin [Nukuzuma et al., 1995]. The plasmid M1-IMRb, derived from IMR-32-adapted JCV, was kindly provided by Dr. Yoshiaki Yogo. TNF-α was obtained from Sigma–Aldrich (St. Louis, MO), and dissolved in a small volume of distilled water.

Quantitation of Cell Proliferation

Cytotoxicity was measured using a Cell Proliferation Kit I (MTT; Roche, Indianapolis, IN) as described in previous studies [Nukuzuma et al., 2012, 2013]. IMR-32 cells in 100 μl medium were plated at a concentration of 5.4×10^4 cells/well into 96-well microtiter plates and incubated in the absence or presence of TNF-α at a final concentration of 25 ng/ml for 3 days at 37°C in a CO_2 incubator, following by the addition of 10 μl (final concentration 0.5 mg/ml) of the MTT labeling reagent to each well. The microtiter plates were incubated for 4 hr. A solubilization solution (100 μl) was added to each well and left to stand overnight in the incubator. The solubilized formazan product was spectrophotometrically quantified using an ELISA reader (Bio-Rad, Hercules, CA) at a wavelength of 550 nm. The reference wavelength was 650 nm.

JCV DNA Transfection

JCV DNA transfection was carried out essentially as described previously [Nukuzuma et al., 2012, 2013]. IMR-32 cells were cultured in 35-mm dishes containing 2 ml complete growth medium (DMEM-10% FBS) until they reached 70–80% confluency. An M1-IMRb clone was used for the DNA replication assay as M1-IMRb showed the highest HA activity in IMR-32 cells [Yogo et al., 1993]. For transfection, 1.0 μg viral DNA, excised from the recombinant plasmid with *Eco*RI, was introduced into the cells using FuGENE 6 transfection reagent (Roche). Endonuclease-cleaved linear viral DNA can be re-circularized after transfection [Yogo et al., 1993].

Real-Time RT-PCR for Expression of the JCV Large T or VP1 Antigen

In order to examine the stimulation of large T or VP1 gene expression in TNF-α-treated IMR-32 cells, a real-time RT-PCR assay was conducted. IMR-32 cells transfected with JCV genome DNA were incubated in culture medium in the absence or presence TNF-α at a final concentration of 25 ng/ml. At 48 hr after TNF-α treatment, the cells were harvested, and total RNA was extracted from three cell cultures using an RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of RNA was treated with DNase I and then introduced into the RT reaction as follows. DNase I-treated RNA was mixed with 40 μl of a reaction mixture containing RT buffer (Toyobo, Osaka, Japan), 1 mM dNTPs, 40 U RNase inhibitor (Toyobo), 200 U ReverTra Ace, as a reverse transcriptase (Toyobo), and 10 pmol oligonucleotide dT_{15} (Roche). The RT mixture was incubated for 10 min at 30°C, 60 min at 42°C, heated to 99°C for 5 min, and then added to the real-time PCR mixture.

For real-time RT-PCR analysis of large T and VP1 gene expression, two sets of PCR primers and TaqMan probes were used according to previous reports [McNees et al., 2005; Nukuzuma et al., 2009]. Real-time PCR analyses were performed in a total volume of 25 μl of 2× TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), 300 nM of each primer, 200 nM TaqMan probe and 5 μl RT product. For the absolute quantification of T and VP1 antigen copies, plasmids containing target sequences were serially diluted from 10^2 to 10^6 copies per reaction and then used as standard DNA templates. Real-time PCR amplification was carried out using an ABI PRISM 7900HI system (Applied Biosystems).

The amplification conditions were as follows: 2 min at 60°C, 10 min at 95°C, 40 cycles of 95°C for 15 s, and 60°C for 15 s. As an endogenous reference, the copy number of the β-actin gene, a housekeeping gene, in each RNA extract was determined by real-time RT-PCR using TaqMan β-actin Control Reagents (Applied Biosystems). The copy number of the T antigen and VP1 mRNA in each sample was normalized against β-actin mRNA copies.

DNA Replication Assay

The DNA replication assay was carried out essentially as described previously [Nukuzuma et al., 2012, 2013]. At 48 hr after TNF-α treatment, the cells were harvested, and low-molecular-weight DNA was extracted from cells according to the Hirt procedure [Hirt, 1967]. The DNA (1 μg) derived from the transfected cells was digested with *Dpn* I and *Bam* HI. The resulting fragments were separated by electrophoresis on a 1.0% agarose gel, transferred onto a nylon membrane (Roche), and hybridized at 65°C in an incubator to digoxigenin-labeled JCV DNA using a DIG DNA Labeling Kit (Roche). Digoxigenin-labeled DNA Molecular Weight Marker was used as the size marker on the gel. Replicated DNA was detected using a DIG Luminescent Detection Kit (Roche), and exposed to film overnight. The exposed X-ray films were then scanned and the intensities of the bands were quantified using ImageJ (National Institutes of Health, Bethesda, MD, http://rsbweb.nih.gov/nih-image/). Relative band intensities were normalized against the intensities of the input DNA and background. The relative intensities were presented with reference to the lowest detectable band intensities by assigning them a value of 1.

DNase Protection Assay of JCV Replication in Transfected IMR-32 Cells

For the DNase protection assay, IMR-32 cells were untreated or treated TNF-α in 25 cm^2 flasks and were resuspended in 250 μl phosphate-buffered saline (pH 7.15) containing 0.2% bovine serum albumin (BSA) and subjected to freeze-thawing three times. The cell lysates were centrifuged at 1,500 rpm for 10 min at 4°C. The resultant supernatants were applied to real-time PCR assay combined with DNase treatment. To eliminate transfected JCV DNA from the cell extracts, 4 μl sample was mixed with 16 μl reaction mixture containing 2U Baseline-Zero DNase and Baseline-Zero buffer (AR Brown, Madison, WI) and incubated for 60 min at 37°C. Next, 15 μl reaction sample was mixed with an equal volume of DNAzol Direct reagent (Molecular Research Center, Cincinnati, OH) and incubated for 10 min at 80°C to inactivate DNase. Then, samples were directly subjected to real-time PCR analysis of the large T antigen [McNees et al., 2005] without any DNA extraction protocol, essentially as described in our previous report [Nakamichi et al., 2011].

Analysis of NF-κB Activation

The activation of the NF-κB pathway was assessed using a NF-κB/p65 ActivELISA Kit (Imgenex, San Diego, CA) according to the manufacturer's instructions. This kit measures the amount of p65 subunit of NF-κB translocated into the nuclei during activation of the NF-κB pathway. IMR-32 cells in 2 ml medium were plated into six-wells plates at a concentration of 8.0×10^5 cells/well and incubated in the absence or presence of TNF-α at a final concentration of 25 ng/ml for 48 hr. The nuclear fraction was extracted according to the lysate preparation manual, and 100 μl (50 μg protein) of each nuclear fraction were then transferred to the wells of an anti-p65 antibody-coated microtiter plate. Next, an anti-p65 antibody, alkaline phosphate-conjugated secondary antibody, and NPP substrate were added to generate a colorimetric signal. The absorbance of the colorimetric signal was measured at a wavelength of 415 nm using a BIO-RAD Model 3550 Microplate Reader (Bio-Rad). For the absolute quantification of p65, the recombinant p65 protein was diluted serially from 15.6 to 1,000 ng/ml per reaction and used as the standard curve.

Statistical Analysis

The significance of intergroup differences was determined using a statistical program and Student's *t*-test.

RESULTS

TNF-α Does Not Induce Any Cytotoxic Effect on IMR-32 Cells

The relative number of live cells was determined by measuring mitochondrial succinate dehydrogenase activity using an MTT assay. The absorbance of formazan products from TNF-α-untreated and -treated cells was 0.711 ± 0.003 and 0.697 ± 0.004, respectively. The enzyme activity of TNF-α-treated IMR-32 cells was slightly lower than that of TNF-α-untreated control cells, but this difference was not statistically significant. This result indicates that TNF-α does not induce a cytotoxic effect on IMR-32 cells under the assay conditions.

TNF-α Stimulates JCV T Antigen Gene Expression in IMR-32 Cells

IMR-32 cells were transfected with JCV genome DNA and incubated in the absence or presence of TNF-α. The expression levels of the JCV large T antigen and VP1 genes were determined by real-time RT-PCR, as described in section Materials and Methods. The mRNA of the large T and VP1 genes from three cultures was quantitatively amplified by real-time RT-PCR. The expression level of the large T antigen gene in TNF-α-treated IMR-32 cells was 6.2-fold higher than that in TNF-α-untreated control cells (Fig. 1), and this difference was statistically

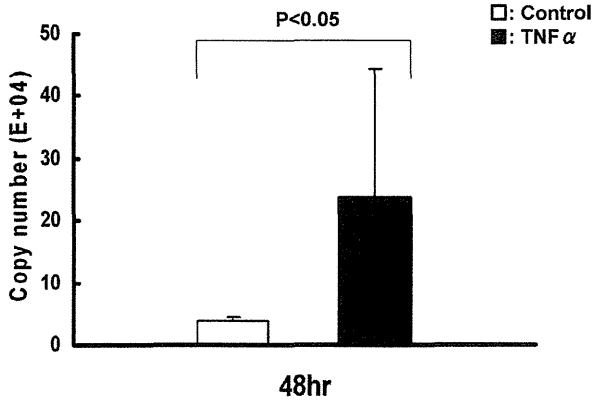

Fig. 1. Stimulation of JCV large T antigen gene expression in IMR-32 cells by TNF-α. The expression levels of the large T antigen gene from three cultures were measured by real-time RT-PCR after incubation for 48 hr in the absence or presence of TNF-α. The linear standard curve was generated from serial dilution with the plasmid M1-IMRb. The amount of large T antigen mRNA in each sample was normalized with reference to the copy numbers of human β-actin mRNA. Data are shown as the mean ± standard deviation of the means. $^*P < 0.05$ (Student's *t*-test).

significant ($P < 0.05$). On the other hand, the expression of the VP1 antigen in TNF-α treated IMR-32 cells was only 2.0-fold greater than that in the control cells (Fig. 2), and this difference was not statistically significant. These results indicate that TNF-α preferentially induces the expression of the large T antigen gene in IMR-32 cells.

TNF-α Stimulates JCV Replication in IMR-32 Cells

IMR-32 cells were harvested at 48-hr post-transfection with JCV genome DNA, and the replicated JCV

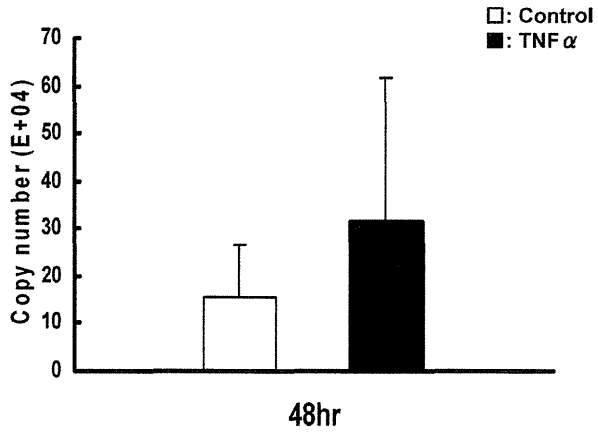

Fig. 2. TNF-α-induced expression of JCV VP1 antigen in IMR-32 cells. The expression levels of the JCV VP1 gene from three cultures were measured by real-time PCR after incubation for 48 hr in the absence or presence of NF-α. The linear standard curve was generated from serial dilution with the plasmid containing the VP1 gene. The amount of VP1 mRNA in each sample was normalized with reference to copy numbers of human β-actin mRNA. Data are shown as the mean ± standard deviation of the means.

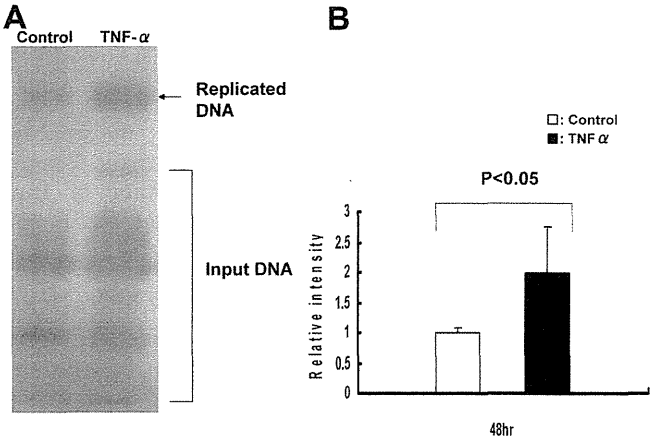

Fig. 3. Stimulation of JCV replication by TNF-α in IMR-32 cells. **A**: IMR-32 cells were transfected with 1.0 μg M1-IMRb and incubated in culture medium in the absence (Control) or presence (TNF-α) of TNF-α. Position of bands representing replicated (5.1 kb, *Dpn* I-resistant) and input (various fragments, *Dpn* I-sensitive) DNA are shown. **B**: The intensities of *Dpn* I-resistant bands were determined as described in Methods section. The columns of the histogram were derived from an analysis of the intensities of the replicated DNAs (*Dpn* I-resistant bands) from three independent experiments using ImageJ. Relative band intensities were normalized against the intensities of the input DNA and background. The relative intensities were presented with reference to the lowest detectable band intensities (Control, 48 hr) by assigning them a value of 1. Data are shown as the mean ± standard deviation of the means. $^*P < 0.05$ (Student's *t*-test).

DNA was detected as described in the Methods section. In TNF-α-treated IMR-32 cells, replicated viral DNA was clearly detected 48-hr post-transfection (Fig. 3A). The columns of the histogram were derived from the densitometry of *Dpn* I-resistant bands from *Bam* HI-digested low-molecular-weight DNA extracted from IMR-32 cells transfected with JCV genome DNA (Fig. 3B). The relative intensities are presented with reference to the lowest detectable band intensities (Control, 48 hr) by assigning the lowest intensities a volume of 1. It can be seen that the relative intensities of JVC genome DNA in TNF-α-treated cells were 2.0-fold greater than those in control cells (Fig. 3B), and this difference was statistically significant ($P < 0.05$). These results indicate that TNF-α stimulates JCV replication in IMR-32 cells.

TNF-α Stimulates JCV Production in IMR-32 Cells

JCV production in TNF-α-treated or -untreated IMR-32 cells was compared using real-time PCR analysis combined with DNase treatment, designated here as a DNase protection assay. Transfected IMR-32 cells were harvested on day 21 after treatment with TNF-α. Extravirion DNAs derived from transfected JCV genome were eliminated with a potent DNase, and the amount of JCV DNA from DNase-protected particles was determined. Under the

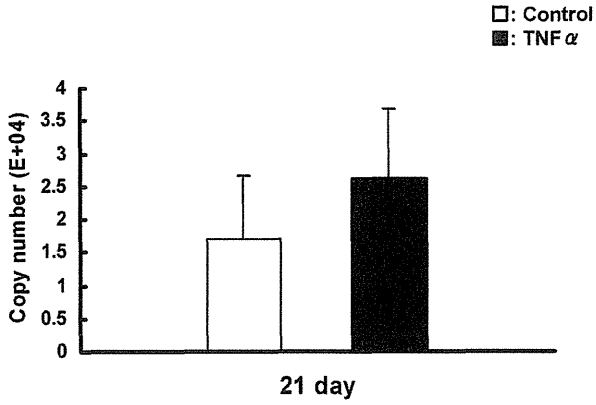

Fig. 4. DNase protection assay of JCV production in TNF-α-treated IMR-32 cells. The cells were transfected with the JCV genome and incubated for 21 days in the absence or presence of TNF-α. The extravirion JCV DNAs were eliminated with a potent DNase (Base-line zero DNase), and the amount of viral genome, which had not been digested by DNase in the particles, was measured by real-time PCR. The linear standard curve was generated from serial dilution with plasmid M1-IMRb. Data are shown as the mean ± standard deviation of the means.

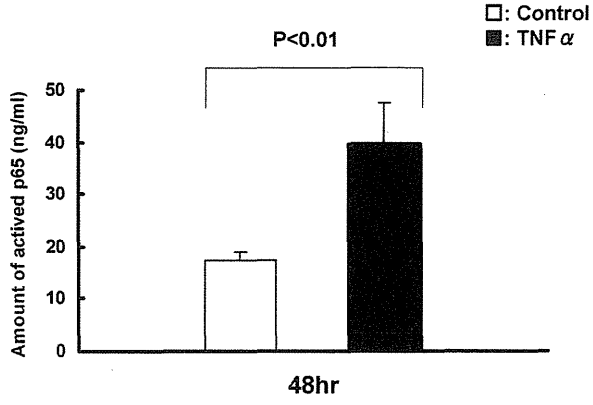

Fig. 5. Stimulation of the NF-κB pathway by TNF-α in IMR-32 cells. IMR-32 cells were plated into six-well plates at a concentration of 8.0×10^5 cells/well in 2 ml medium and incubated in the absence (Control) or presence (TNF-α) of TNF-α for 48 hr. The nuclear fraction was extracted from each of the four cultures. The amount of p65 was measured using ELISA. Data are shown as the mean ± standard deviation of the means. $^*P < 0.01$ (Student's t-test).

conditions used, at least 7×10^9 copies of the transfected JCV genome could be digested by DNase, while the replicated JCV particles remained intact (data not shown). The results of real-time PCR show that the amount of JCV DNA derived from virus particles in TNF-α-treated IMR-32 cells was 1.5-fold greater than that in control cells, although this difference was not statistically significant (Fig. 4).

TNF-α Stimulates the NF-κB Pathway

Activation of the NF-κB pathway in TNF-α-treated IMR-32 cells was quantified by measuring the nuclear translocation of NF-κB/p65, as described in section Materials and Methods. The amount of p65 in TNF-α-treated cells was 2.3-fold greater than that in untreated cells, and this difference was statistically significant ($P < 0.01$) (Fig. 5). These results indicate that TNF-α stimulates the activation of the NF-κB pathway in IMR-32 cells.

DISCUSSION

AIDS–PML has become prevalent and is found in approximately 4% of all AIDS patients [Major et al., 1992]. The high incidence of PML among individuals with AIDS suggests that PML is particularly associated with AIDS. HIV-1 induces the production of pro-inflammatory cytokines such as TNF-α in the CNS [Benveniste, 1994; Yeung et al., 1995; Kaul et al., 2005]. In this study, the effects of TNF-α on viral gene expression were examined in IMR-32 cells transfected with JCV DNA. The plasmid M1-IMRb contains the viral genome of the JCV mutant strain, which can productively replicate in IMR-32 cells. This mutant JCV was obtained from serial passaging of the JCV Mad-1 strain in IMR-32 cells. The viral genome of M1-IMRb has deletions of the TATA sequence distal from the origin of replication within the regulatory region, leading to a similar sequence to that of the Mad-4 strain. However, deletion endpoints, relative to Mad-1, were not identical between Mad-4 and M1-IMRb. The expression level of the JCV large T gene in IMR-32 cells was increased following TNF-α treatment. Since the NF-κB pathway in IMR-32 cells was activated in the presence of TNF-α, it can be speculated that TNF-α-mediated activation of the NF-κB pathway leads to the upregulation of JCV large T expression. The results of the DNA replication assay and real-time RT-PCR in the transfection experiment show that the replication of JCV genome DNA in IMR-32 cells was increased in the presence of TNF-α. Thus, the upregulation of large T expression is necessary for viral DNA replication.

In a previous study by other investigators, the effects of cytokines, TNF-α, IL-6, IL-1β, and TGF-β, on the transcription of JCV early and late genes were examined using reporter assays. Each reporter assay included phorbol 12-myristate 13-acetate (PMA) as a positive control. TNF-α stimulated the early promoter as strongly as PMA and TGF-β, IL-6 had a minimal effect, and the effect of IL-1β was intermediate. The expression of the late promoter of JCV in TNF-α-treated cells was statistically significantly higher than in cells treated with other cytokines [Wollebo et al., 2011]. It was suggested that TNF-α stimulates JCV transcription in both the early and late phases of infection in the human oligodendroglioma cell line [Wollebo et al., 2011]. These studies indicated a relationship between TNF-α and AIDS–PML pathogenesis. It has been noted that TNF-α stimulates the transcription of the JCV reporter construct containing a NF-κB binding site (κB element) [Wollebo et al.,

2011]. However, it has also been reported that TNF-α does not stimulate JCV transcription in human glial cells [Atwood et al., 1995] as the JCV reporter construct in the study did not contain a κB element.

NF-κB is a transcriptional factor that can be activated by TNF-α and, in turn, activates the expression of HIV-1 in T cells [Nabel and Baltimore, 1987; Fiers, 1991; West et al., 2001]. The κB element also regulates JCV promoter activity in CNS-derived cells [Ranganathan and Khalili, 1993]. When the amounts of p65 were increased in the presence of nuclear factor of activated T cells 4 (NFAT4), there was a synergistic enhancement of JCV early transcription [Wollebo et al., 2012]. NFAT4 has a role as a cell-signaling phosphatase in neurons and glia [Ho et al., 1994; Graef et al., 1999]. Further analyses, such as the gene expression of NFAT4, need to be conducted to better understand TNF-α mediated JCV replication in IMR-32 cells.

The regulation of JCV replication by TNF-α in AIDS-related PML is currently unknown. In fact, there have been no reports of TNF-α-stimulated JCV replication in culture cells to date because a previous report showed the effect of TNF-α on JCV early and late transcription in reporter assays [Wollebo et al., 2011].

While astrocytes are targets of HIV-1 infection, they are semi-permissive of JCV infection as they allow the expression on T antigen and late gene expression to a limited extent without virus production in vivo. Previous studies revealed that IMR-32 cells transfected with M1-IMRb could be transferred repeatedly accompanied by continuous JCV production. Since it is difficult to propagate JCV in astrocytes, IMR-32 cells are useful for studying the role of TNF-α in JCV production. In this study, JCV production in IMR-32 cells transfected with M1-IMRb was compared using real-time PCR analysis combined with DNase treatment. Extravirion DNAs derived from the transfected JCV genome were eliminated with a potent DNase, and the amount of JCV DNAs from the DNase-protected particles was determined. Further developments are needed to establish a JCV production system using astrocytic cell lines.

A previous report based on in situ hybridization analysis showed that TNF-α did not increase the multiplication of JCV in human fetal glial cells infected with JCV [Atwood et al., 1995]. The results of the DNA replication assay in this study were not identical with those of in situ hybridization analysis. This difference is not inconsistent as the DNA replication assay used in this study is able to distinguish between newly replicated JCV DNA and transfected DNA in IMR-32 cells. On the other hand, no significant increase in VP1 gene expression or virus multiplication was observed in real-time RT-PCR analysis and the DNase protection assay, respectively. This is in contrast to the effect of TNF-α on JCV replication through the upregulation of large T antigen expression in IMR-32 cells. These results suggest that TNF-α mainly induces the gene expression of the large T antigen, which is necessary for JCV replication in IMR-32 cells, leading to the stimulation of viral genome replication in the early stage of infection. Thus, TNF-α may induce JCV reactivation. Many important aspects of PML pathogenesis remain unclear, including the upregulation of viral transcription and replication. As TNF-α stimulated the expression of large T antigen and viral replication, this cytokine may contribute to JCV propagation in AIDS-related PML. This finding may contribute to understanding the pathogenesis of AIDS-related PML.

ACKNOWLEDGMENTS

We thank Dr. Yoshiaki Yogo for kindly providing the plasmid containing the JCV M1-IMRb strain genome, and Mr. Shozo Isoda for continuous encouragement.

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Arch Virol
DOI 10.1007/s00705-014-1988-4

ORIGINAL ARTICLE

High-resolution melting analysis for mutation scanning in the non-coding control region of JC polyomavirus from patients with progressive multifocal leukoencephalopathy

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Received: 20 September 2013 / Accepted: 12 January 2014

Abstract JC polyomavirus (JCV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease. JCV isolates from PML patients have hypervariable mutations in the noncoding control region (NCCR) of the viral genome. Although nucleotide sequencing analysis of NCCR mutation is useful for the confirmation of PML diagnosis and basic studies examining JCV variants, it is often labor-intensive, time-consuming, and expensive. This study was conducted to evaluate the feasibility of a high-resolution melting (HRM) analysis technique for the rapid and low-cost scanning of NCCR mutations. The real-time PCR-HRM assay was developed with a pair of primers targeting the NCCR, and mutational patterns of NCCRs were compared using sequence-confirmed JCV DNA clones and CSF DNAs from PML patients. The NCCR patterns of DNA clones of the archetype JCV and PML-type variants could be differentiated by PCR-HRM. The mutational patterns of the rearranged NCCR clones were similar to those of JCV variants in the original CSF specimens as judged by nested PCR-HRM using pre-amplified targets. In addition, nested PCR-HRM could distinguish NCCR mutations in the JCV DNAs from each specimen at the patient level. These results indicate that the HRM-based assay affords a valuable technique for PML diagnosis and a versatile tool for the rapid scanning of NCCR mutations.

Introduction

Progressive multifocal leukoencephalopathy (PML) is a rare but fatal demyelinating disease of the central nervous system (CNS) caused by JC virus (JCV), a small DNA virus belonging to the family *Polyomaviridae*, genus *Polyomavirus* [1–3]. Humans are infected with JCV asymptomatically during childhood, resulting in persistent infection throughout their life. From 50 to 90 % of adults have been reported to be serologically positive for JCV [1–4]. However, in some severely immunocompromised patients, JCV reactivates and causes a lytic infection in the oligodendrocytes, leading to PML [1–4]. PML develops in HIV-positive patients as well as in those that are immunodeficient due to hematological malignancies, chemotherapy, transplantation, lymphocyte depletion or the treatment of autoimmune disorders with immunosuppressive agents, including monoclonal antibodies, such as natalizumab, rituximab, and efalizumab [1–3, 5, 6].

The detection of JCV DNA in the cerebrospinal fluid (CSF) by PCR is a reliable and less-invasive diagnostic marker of PML [7, 8]. The rapid and specific quantification of JCV DNA using a real-time PCR technique has become the current diagnostic standard [5]. However, because of its sensitivity, real-time PCR has a risk of false-negative results due to DNA contamination of samples [9]. Clinical isolates of JCV can be classified into two groups on the basis of sequence divergence in the non-coding control region (NCCR; also referred to as the regulatory region or transcription control region) of the viral genome [10–12]. Nonpathogenic JCV strains isolated from the urine of healthy individuals contain a consistent NCCR sequence known as the archetype [13]. In contrast, JCV isolates from PML patients are characterized by hypervariable mutations within the NCCR [12, 14]. The changes in the NCCR sequences are thought to be related to the activation of virus replication during disease progression [12, 14, 15]. These mutated sequences are thought to be derived from the archetype NCCR via deletions and/or duplications [16, 17], leading to the alteration of promoter activity [18, 19].

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Published online: 25 January 2014