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AZT誘発ミトコンドリア機能障害に対する分子治療方法の開発

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厚生労働科学研究費補助金(エイズ対策研究事業)
(総合)研究報告書
AZT誘発ミトコンドリア機能障害に対する分子治療方法の開発

研究代表者 佐藤 岳哉 東北大学大学院医学系研究科・助教

研究要旨:抗HIV薬のアジドチミジン(AZT)は、HIVに対する有効な作用を発揮する。その反面、重篤な副作用、心筋ミオパチーを誘発するが、その分子機構は不明である。本研究では、AZT 代謝に関与する酵素チミジル酸キナーゼ(tmpk)の変異型を発現するラット心筋培養細胞を用いて、AZT の細胞に対する効果を検討した。種々の検討の結果、AZT 代謝物のうち活性化体である AZT3 リン酸がミトコンドリア機能障害を強く誘発することが明らかとなった。さらに AZT3 リン酸が誘発するミトコンドリア機能障害を低濃度のシクロスポリン A が効果的に防止することを見いだした。

分担研究者 柳澤 輝行
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A. 研究目的

抗 HIV 薬として用いられているアジドチミジン(AZT)代謝物が誘発するミトコンドリア機能障害の詳細な分子機構の詳細な解析から、ミトコンドリア機能障害を誘発する責任分子(群)すなわち AZT 代謝物が標的とする分子(群)を同定することを目的とする。さらにこの副作用を持たない新規薬物、あるいは、副作用に対する保護作用を示す薬物をスクリーニングすることにより、HAART 治療における AZT 誘発ミトコンドリア機能不全症を防ぐ分子標的薬の開発を目指す。

B. 研究方法

1) 遺伝子導入用ベクター入手と組換えウィルス作成および細胞への遺伝子導入

Jeffrey A. Medin 教授(トロント大学・カナダ)より、チミジル酸キナーゼ (Tmpk)の野生型あるいは変異型 cDNA を持つレンチウィルスベクターの分与を受け、これを使用した。入手したレンチウィルスベクターを用いて、組換えウィルスを作製した。培養ラット心筋細胞 H9c2 にこのウィルスを感染させることで tmpk 遺伝子導入を行った。これらの細胞における tmpk 遺伝子の発現を確認するために、ドイツ Maxplank 研究所の Manfred Konrad 博士から提供された抗ヒト tmpk 抗体を用いる Western blot 法による解析を行った。

2) 細胞内 ATP 量変化を目印にした AZT 感受性試験

非遺伝子導入細胞、tmpk 野生型、変異型遺伝子導入細胞について、種々の濃度の AZT 存在下で 4 日間培養後、ATP 量を Cell Titer Glo キット (Promega)を用いて定量した。

3)ミトコンドリア内膜膜電位測定による AZT 誘発ミトコンドリア機能障害の評価

AZT 処置後に細胞内に蓄積する AZT 代謝物のミトコンドリア機能に対する影響を検討するために、1 mM 存在下、4 日間培養した細胞をミトコンドリア内膜の膜電位感受性の蛍光プローブ JC-1 を用いて染色後、JC-1 の蛍光強度を蛍光プレートリーダーを用いて測定した。

4) AZT 代謝物が誘発するアポトーシス誘導効果

の評価
ミトコンドリア機能障害が起きると細胞は、アポトーシスを起こすことが知られている。そこで、AZT処置後の細胞においてアポトーシスが起きているかどうかについて、アポトーシスの初期に細胞膜表面に発現が増加するホスファチジルセリンに特異的に結合する蛍光標識アネキシンVを用いて、細胞を染色し、細胞に結合したアネキシンVの蛍光強度をフローサイトメトリにより測定を行った。

5) AZT および類薬の細胞に対する影響の比較検討

上記細胞の AZT および類薬感受性を調べるために種々の濃度の薬物存在下で 4 日間培養後、細胞の生存率をルシフェラーゼ法で定量した。

6) AZT 誘発ミトコンドリア機能障害を防止する薬剤の効果の検討

AZT(100 μM)が誘発するミトコンドリア機能不全を介する細胞死に対する保護作用をもつ可能性のある薬剤として Cyclosporin A (CsA)を 0.01 μM～1 μM の終濃度で添加し、一定時間細胞を培養後、細胞内 ATP 量をルシフェラーゼ法により定量した。

7) AZT 代謝物の分子型の違いによる細胞に対する影響の評価

細胞内に蓄積する AZT 代謝物に関する HPLC を用いる検討において、tmpk cDNA を導入した細胞では、AZT-3 リン酸 (AZTTP) が蓄積していたが、その前段階であるAZT-2リン酸 (AZTDP) もまた蓄積していた。AZT を細胞に添加すると両者が細胞内に蓄積し、細胞死を誘導する。このどちらがより強い細胞死誘導活性を持つかを検討するために、AZTDP から AZTTP への変換過程を担うヌクレオシド二リン酸キナーゼ(nucleoside diphosphate kinase: NDK)に対するRNAiベクターを用いて、主にAZTDPが蓄積する細胞系を作製した。Tmpk 遺伝子導入細胞系(主に AZTTP が蓄積)、今回作製した細胞系(主に AZTDP が蓄積)、あるいは遺伝子導入していない細胞(主に AZT-1 リン酸(AZTMP)が蓄積)を用い、200 μM AZT 存在下で一定時間培養を行い、アポトーシス誘導率(アネキシンV を用いて測定)および細胞の生存率(ルシフェラーゼ法)を定量した。

(倫理面への配慮)

本研究において、倫理面において配慮が必要とされる研究は行わない。また、本研究においては、安全対策を必要とするレンチウィルスベクターの使用が含まれているが、申請者らはすでにこのウィルスベクター系を使うことに対する十分な安全対策を施した遺伝子組み換え実験計画を東北大学遺伝子組換え実験安全専門委員会に申請し、承認済みである。この試験計画を試行するにあたり、試験に使用した大腸菌、細胞および組換え DNA 分子は、オートクレーブ等により不活化して廃棄する。

C. 研究結果

(1)遺伝子導入細胞における導入遺伝子発現の確認

分与されたウィルスベクターの配列確認と組み換えウィルス作製を行った。作製したレンチウィルスベクターを用いて、tmpk 遺伝子を導入した H9c2 細胞における導入遺伝子の発現をウサギ抗ヒト tmpk 抗体を用いる Western blot 法で確認した。非遺伝子導入細胞である親株の細胞では、ヒト tmpk の発現は、確認されなかったのに対し、tmpk 野生型あるいは変異型遺伝子を導入した細胞においては、ヒト tmpk の分子量に相当するタンパク質のバンドが確認された。

(2)AZT 処置後の ATP 量の変化の詳細な検討

Tmpk 変異型遺伝子発現細胞においては、AZT 濃度依存的な ATP 量の減少が観察されたが、対照群(親株、および tmpk 野生型遺伝子発現細胞)では、それがみられなかった。また、tmpk 変異型遺伝子導入細胞は、10 μM 以上の濃度の AZT 添加により、AZT 濃度依存性かつ時間依存的な顕著な細胞内 ATP 量の減少がみ

られた。一方、対照群では、100 μM 以上の AZT 存在下において、有意な細胞内 ATP 量の低下が観察された。以上より、tmpk 変異型遺伝子発現細胞は、AZT 感受性が対照群よりも亢進していることを確認した。

(3) AZT 処置後のミトコンドリア膜電位変化の検討

1 mM AZT 存在下、4 日間培養した細胞を JC-1 で染色し、JC-1 の蛍光強度を測定した。Tmpk 変異型遺伝子発現細胞においては、AZT 処置により顕著なミトコンドリア内膜の膜電位低下が認められた。一方、対照群ではそれが認められなかった。

(4) AZT 処置後のアポトーシス誘導に関する検討

1 mM AZT 存在下、4 日間培養した細胞についてアポトーシスの誘導を検討した。Tmpk 変異型遺伝子発現細胞では、AZT 処置によるアポトーシス誘導が未処置細胞に比して顕著かつ有意な亢進を示した。それに対し、対照群の細胞のうち、tmpk 野生型細胞を AZT 処置後のアポトーシス誘導は親株のそれよりも増加傾向が見られたが、有意ではなかった。

(5) AZT および類薬による細胞死誘導効果の比較

AZT および類薬として d4T, ddC, ddI, Lamivudine を種々の濃度で使用した。4 日間薬剤の存在する状態で培養後の、細胞の生存率測定を行った。その結果、Tmpk 遺伝子導入細胞に対する細胞死誘導効果は、AZT がもっとも強く d4T, Lamivudine, ddI, ddC の順であった。

(6) AZT 誘発ミトコンドリア機能障害を防止する薬剤の効果の検討

今回使用した濃度の CsA 自身によるミトコンドリア機能障害による細胞の ATP 含量の低下効果は、観察されなかった。次に 100 μM AZT 存在下で種々の濃度の CsA を共存させ 4 日間培養を行い、その後細胞の ATP 含量をルシフェラーゼ法で測定した。その結果、CsA が存在しない状態では、細胞の ATP 含量がコントロールに比して 40%減少したのに対し、0.01 μM の CsA 濃度では、それが 25%の減少に止まった。それ以上の用量の CsA では、細胞の ATP 含量は CsA 0.01 μM 用いたものよりも若干減少するが、CsA を用いない対照群と比して有意に細胞の ATP 含量は亢進した。

(7) AZT 代謝物の分子型の違いによる細胞に対する影響の評価

NDK に対する RNAi ベクターを導入した細胞においては、NDK のタンパク質発現レベルは対照群に比して、80%に減少した。次に、遺伝子導入していない細胞(親株)、tmpk-cDNA 導入細胞(Tmpk)、および tmpk-cDNA 導入細胞に NDK に対する RNAi ベクターをさらに導入した細胞(NDK/RNAi)を用いて、200 μM AZT 存在下、2 日間培養後、細胞の生存率を MTT 法で定量した。その結果、親株では共存する AZT により細胞の生存率が AZT を添加しない対照に比して 12 % 減少し、Tmpk では、41 %、NDK/RNAi では 53%、それぞれ細胞の生存率が低下した。また、アポトーシス誘導率については、親株では共存する AZT により、対照に比して 1.9 倍、Tmpk では、2.6 倍、NDK/RNAi では、5.5 倍増加する結果となった。

D. 考察

(1) 遺伝子導入細胞における導入遺伝子発現の確認 Tmpk 遺伝子導入を行った細胞では、導入遺伝子の発現が確認された。ラット由来の親株における内在性 tmpk の発現は確認されなかったが、これは用いた抗体の特異性(ヒト tmpk 特異的)によるものと考えている。

(2) AZT 処置後の ATP 量の変化の詳細な検討

Tmpk 遺伝子発現細胞を 1 mM AZT 存在下で 4 日間培養後、細胞内 ATP 量を測定したところ、tmpk 変異体遺伝子発現細胞において、ATP 量の顕著な低下がみられたのに対し、対照群ではそれがみられなかった。これは、発現させた tmpk 変異体蛋白質により、AZT が活性化体 AZTTP へ変換され、ミトコンドリア機能障害を誘

発したためと考えられた。

(3) AZT処置後のミトコンドリア膜電位変化の検討

AZT 誘発ミトコンドリア機能障害を確認するために、AZT 処置後の細胞におけるミトコンドリア機能障害の指標としてミトコンドリア内膜膜電位を測定した。Tmpk 変異型遺伝子発現細胞においては、AZT 処置により顕著なミトコンドリア内膜の膜電位低下が認められた。一方、対照群ではそれが認められなかった。これは AZT 代謝物のうち AZTTP が、対照群の細胞において蓄積すると考えられる AZT 中間代謝物 AZTMP よりもミトコンドリア機能を強く障害することを示すものである。

(4) AZT 処置後のアポトーシス誘導に関する検討

AZT 処置によりミトコンドリア機能障害が誘発されると、細胞のアポトーシスが進行すると考えて、AZT 処置後の初期アポトーシス亢進について検討した。その結果、予測したとおり Tmpk 変異型遺伝子発現細胞においては、AZT 処置により有意かつ顕著なアポトーシス亢進が認められた。一方、対照群ではそれが認められなかった。これらの結果から、AZT 誘発ミトコンドリア機能障害において、AZT 代謝物のうち、AZTTP が AZTMP よりも強くそれを誘導するということを明らかにした。従来は、AZT 代謝物のうち AZTMP がミトコンドリア機能障害を誘発すると考えられていた。しかしながら、今回確立した細胞系を用いた検討では、それとは異なる結果を得た。現在の評価に要する時間は、約 4 日であるが、従来の AZT 代謝物の影響を評価する際には 1 カ月以上の時間を必要とする。今回の検討結果と、従来の評価結果を比較、検討を行うためには、本細胞系を用い、より低濃度 AZT 処置後のより長期のミトコンドリア機能障害について検討をする必要がある可能性があると考えている。

(5) AZT 誘発ミトコンドリア機能障害を防止する薬剤の効果の検討

Tmpk 変異体遺伝子導入細胞を AZT で処置するとミトコンドリアの機能障害が誘発され、ミトコンドリア内膜の膜電位消失、アポトーシスの進行が起きることを上記で確認した。ミトコンドリア内膜の膜電位形成に重要な役割をもつ mitochondrial permeablility transition pore (mPTP)の開口抑制作用をもつ CsA を用いた。CsA は、mPTP の開口を抑制することで、ミトコンドリア内膜の膜電位消失を抑制し、その結果、AZT誘発細胞死の抑制を行うことができると予測した。CsA は、通常免疫抑制剤として使用されるが、今回用いた CsA の濃度は免疫抑制剤として使用する濃度の 1／1000 程度である。今回検討した CsA 濃度においては、AZT 誘発ミトコンドリア機能障害による細胞の ATP 含量低下を効果的に抑制することができた。

(6) AZT 代謝物の分子型の違いによる細胞に対する影響の評価

AZT 代謝物の中で、従来 AZTMP が細胞毒性を持つことが知られていた。本研究で Tmpk 変異体遺伝子導入細胞を構築したことで、AZTTP を効率よく生ずる系を構築し、AZTTP の方が AZTMP よりも細胞毒性が強いことを明らかにしてきた。AZTDP の細胞毒性については、不明であったので、tmpk 変異体遺伝子導入細胞にさらに NDK に対する RNAi ベクターを導入した細胞(NDK/RNAi)を作成した。AZT が代謝されて生ずる各代謝物(1リン酸体、2リン酸体、3リン酸体)が細胞に与える影響を比較するために、遺伝子導入していない細胞(以後親株と記す:主に AZTMP が蓄積する)、tmpk 変異体遺伝子導入細胞(以後 Tmpk と記す:主に AZTTP が蓄積する)、および NDK/RNAi (主に AZTDP が蓄積する)を用いて AZT 処理後の細胞の生存率の検討を行った。これらの細胞群を用いた検討の

結果、細胞内に蓄積する AZT 代謝物の中で AZTDP が AZTTP よりも細胞毒性が若干であるが強いということを明らかにした。

E. 結論

本研究の検討結果から、AZT代謝物が誘発するミトコンドリア機能障害は、従来原因物質として考えられていた AZTMP ではなく、活性化体もしくは活性化中間体の AZTTP あるいは AZTDP であることを明らかにした。さらに AZT 誘発ミトコンドリア機能障害を低濃度の CsA が抑制できることを明らかとした。本研究で確立した tmpk 遺伝子発現 H9c2 細胞は、AZT 代謝物誘発ミトコンドリア機能障害およびアポトーシス誘導の分子機構を詳細に検討することができる有用なものである。今後、これを用いて AZT などの NRTI により誘発される心筋ミオパチーの詳細な分子機構の検討および、それを基盤として NRTI 誘発心筋ミオパチーに対する分子標的治療薬の開発を進めていく必要があると考えている。

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新生マウスに対する VEGF 前投与は遺伝子治療効果を改善する新生マウスに対するVEGF前投与は遺伝子治療効果を改善する、第 83 回日本薬理学会年会、March 16-18, 2010, 大阪

G. 知的財産権の出願・登録状況

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

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別紙４

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

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
Sato T, Neschadim A, Konrad M, Fowler DH, Lavie A, Medin JA.	Engineered human tmpk/AZT as a novel enzyme/prodrug axis for suicide gene therapy.	Molecular Therapy	15 (5)	962-970	2007
Takemoto J, Masumiya H, Nunoki K, **Sato T**, Nakagawa H, Ikeda Y, Arai Y, **Yanagisawa T**	Potentiation of potassium currenta by β-adrenoceptor agonists in human urinary bladder smooth muscle cells: a possible electrical mechanism of relaxation.	Pharmacology	81	251-258	2008
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Engineered Human tmpk/AZT As a Novel Enzyme/Prodrug Axis for Suicide Gene Therapy

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Gene therapy and stem cell transplantation safety could be enhanced by control over the fate of therapeutic cells. Suicide gene therapy uses enzymes that convert prodrugs to cytotoxic entities; however, heterologous moieties with poor kinetics are employed. We describe a novel enzyme/prodrug combination for selectively inducing apoptosis in lentiviral vector–transduced cells. Rationally designed variants of human thymidylate kinase (tmpk) that effectively phosphorylate 3′-azido-3′-deoxythymidine (AZT) were efficiently delivered. Transduced Jurkat cell lines were eliminated by AZT. We demonstrate that this schema targeted both dividing and non-dividing cells, with a novel killing mechanism involving apoptosis induction via disruption of the mitochondrial inner membrane potential and activation of caspase-3. Primary murine and human T cells were also transduced and responded to AZT. Furthermore, low-dose AZT administration to non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice injected with transduced K562 cells suppressed tumor growth. This novel suicide gene therapy approach can thus be integrated as a safety switch into therapeutic vectors.

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INTRODUCTION

Leukemic transformation in gene therapy patients[1] has led to concern regarding the safety of integrating viral vectors. Nonetheless, these vectors offer efficient transduction and long-term gene expression. Research is directed toward increasing vector safety and reliability. A promising approach is to establish control over the fate of transduced cells. Incorporating an effective suicide gene can ensure that any malignant clones arising from insertion of the recombinant retroviral vector can be removed. Likewise, such control could be used for potential complications of embryonic stem cell transplantations, including therapy for malignant teratomas as developed in one recent study.[2]

The success of acyclovir against herpes simplex virus (HSV) infections[3] has engendered a strategy to kill tumors by delivering genes for drug-converting enzymes into malignant cells.[4] HSV thymidine kinase (HSV-tk) is involved in converting the prodrug ganciclovir (GCV) into its tri-phosphorylated form. GCV-triphosphate (TP) causes DNA chain termination during replication, ultimately leading to cell death.[5] In recent years, HSV-tk and mutants have become the most commonly employed enzymes for suicide gene therapy.[6,7] However, weaknesses of this strategy are the foreign origin of the transgene and the fact that overexpression of HSV-tk may simply redirect the rate-limiting step in the conversion to GCV-TP to the second enzyme in the pathway: guanylate kinase. Evidence is emerging, in particular from clinical trials,[8,9] that immune responses against HSV-tk limit the persistence of transduced cells. Successful suicide gene therapy, especially as a safety component for long-term correction of inherited diseases, for example, will thus require the expression of a non-immunogenic protein, either an overexpressed human enzyme or a minimally modified variant thereof.

The prodrug 3′-azido-3′-deoxythymidine (AZT) is converted through phosphorylation into AZT-triphosphate (AZT-TP).[10] AZT-TP inhibits replication of human immunodeficiency virus (HIV),[11,12] and to a lesser extent DNA replication in eukaryotic cells.[13] Safety profiles for this compound are well known. The rate-limiting step in the conversion of AZT to the toxic AZT-TP form is the phosphorylation of AZT-monophosphate (AZT-MP) to AZT-diphosphate (AZT-DP). This is catalyzed by cellular thymidylate kinase (tmpk), which has a low enzymatic efficiency for AZT-MP.[14] We predicted that increased AZT-TP concentrations, achieved by a strategy that accelerates the monophosphate-to-diphosphate conversion of the prodrug, would yield potent cytotoxicity. To improve the processing of AZT-MP to AZT-DP, we employed minimally modified tmpk mutants (F105Y and R16G-Large Lid (R16GLL)) with up to 200-fold enhanced activity for AZT-MP.[15,16] The F105Y variant encompasses a single substitution of the phenyalanine at position 105 to a tyrosine residue. The R16GLL variant was generated by exchanging 11 amino acid residues in the wild-type (WT) sequence with 13 residues from the *Escherichia coli* tmpk ortholog.

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The safety aspect of suicide gene therapy relies on efficient delivery and stable expression of the cytotoxic effector. Lentiviral vectors (LVs) can transduce a wide range of dividing and non-dividing cell types with high efficiency, conferring stable, long-term transgene expression.[17,18] Reliable suicide gene therapy also requires that the overwhelming majority of transduced cells express the suicide gene. This can be ensured by introducing a cell-surface marker gene.[19] Transduced cells can be enriched based on expression of this marker. A cell-surface marker should be inert in itself, devoid of signaling capacity, and non-immunogenic.[20] Previously we have used a variety of cell-surface markers in this context: human CD24,[21] murine heat-stable antigen,[22] human CD25,[23] and a truncated form of LNGFR.[24]

Although human CD25 has been effective for our murine studies,[18,25] it is not useful for T-cell applications because endogenous expression is up-regulated in that population upon activation. Overexpression of a truncated form of LNGFR has promoted transformation of myeloid cells in an unusual, highly context-dependent manner.[26] Here, we adapt a novel truncated form of human CD19 (huCD19Δ) as our marker. CD19 is a 95-kd glycoprotein of the immunoglobulin superfamily that complexes with CD21, CD81, and Leu-13, which collectively function to modulate the activation threshold of the B-cell receptor.[27,28] As expression of CD19 and CD21 is restricted to B-cell lineages,[29] it is suitable for use in murine and human T cells. To further decrease signaling capacity, we have deleted the cytoplasmic tail[30] of CD19.

We have evaluated a novel prodrug/enzyme combination for suicide gene therapy. Catalytically improved variants of human tmpk along with huCD19Δ were efficiently delivered by novel bicistronic LVs, and selective clearance of cells *in vitro* and *in vivo* in response to increasing AZT concentrations was evaluated systematically. We present analysis of AZT sensitivity in transduced cells and further demonstrate a novel cytotoxic mechanism: that increased accumulation of intracellular AZT-TP decreases cell viability, partly owing to activation of a mitochondria-mediated apoptosis pathway. This system thus describes a practical choice for suicide gene therapy that can help to establish the next generation of safer integrating viral vectors. In addition, this schema can also be used to endow stem cells destined for utility in clinical transplantation with a reliable safety control system.

RESULTS

Synthesis of novel suicide LVs expressing modified tmpks and huCD19Δ

Figure 1 shows schematics of the recombinant LVs we constructed. Jurkat cells were transduced a single time (multiplicity of infection = 10). Five days later, huCD19Δ expression was examined. Although no huCD19Δ expression was observed on non-transduced (NT) Jurkat cells, more than 95% of transduced cells showed strong huCD19Δ expression (data not shown). Individual cell clones were isolated by limiting dilution and flow cytometry. The mean fluorescent intensity of huCD19Δ expressed on transduced cell clones showed similar values to each other (data not shown). Transduced cell clones were also examined by flow cytometry after intracellular immunostaining with rabbit anti-human tmpk. Basal expression of tmpk was detected in NT Jurkat cells. Cells transduced with LVs/tmpks showed increased tmpk expression, up to five times higher than in controls (data not shown). Western blots on cell lysates further demonstrated that expression levels of WT or variant forms of tmpk were similar in the transduced cell clones (data not shown).

Determination of intracellular AZT metabolites in LV/tmpk-transduced cells

To confirm functionality of the tmpk mutants, we measured the intracellular amounts of AZT metabolites using reverse-phase high-performance liquid chromatography (HPLC). Cells expressing the R16GLL mutant tmpk efficiently converted AZT-MP into AZT-DP and then, by other kinases, into AZT-TP, whereas the main metabolite that accumulated in the NT-Jurkat cells was AZT-MP (**Figure 2**). Interestingly, we observed no significant increases in the accumulation of AZT-TP or induction of cell death (see below) in the cells overexpressing WT tmpk itself (data not shown). This demonstrates the importance of using engineered enzymes optimized for AZT-MP phosphorylation. The ratio of AZT-TP to AZT-MP in each cell population was calculated from the values of the area under the curve of each chromatogram. **Figure 2** shows that overexpression of the R16GLL and F105Y mutants induced a pronounced increase in the AZT-TP/AZT-MP ratio compared with controls. Mitochondrial fractions were enriched from control LV-internal ribosome entry site (IRES) huCD19Δ and test LV-R16GLLtmpk-IRES-huCD19Δ-transduced cell clones and analyzed using HPLC for levels of AZT metabolites. In the control cells, AZT-MP, AZT-DP, and AZT-TP were observed at appreciable levels. In contrast, only AZT-DP and AZT-TP were observed in the mitochondria of the cells expressing the R16GLL tmpk mutant (data not shown).

AZT sensitivity of LV/tmpk-transduced cells

By itself, transduction of Jurkat cells with LVs engineering expression of controls or our modified suicide genes and huCD19Δ did not affect proliferation (data not shown). To examine the effect of exposure to AZT on cell viability, we incubated the tmpk-expressing cells with increasing concentrations of AZT and after 4 days determined the percentage of living cells (**Figure 3**). Transduced cells expressing the AZT-MP catalytically-optimized tmpk mutants F105Y or R16GLL were minimally viable upon addition of AZT in a dose-dependent manner ($P < 0.0001$). In contrast, limited cell killing, even at high doses of AZT up to 1 mM, was observed in the control cells.

As we observed the formation of nuclear apoptotic bodies by 4′-6-diamidino-2-phenylindole staining in the tmpk mutant–expressing cells treated with prodrug (data not shown), we speculated that active metabolites of AZT induced cell death by apoptosis. To confirm this, we stained the AZT-treated tmpk-expressing cells with Annexin V and performed flow cytometric analyses. In response to 100 μM AZT exposure, early apoptotic indices of cells expressing the F105Y and the R16GLL tmpk mutants were significantly increased ($P < 0.0001$) compared with those in the absence of AZT treatment (9.5 ± 0.8–fold and 8.3 ± 0.4–fold increases, respectively).

HSV-tk-mediated cell killing requires cellular proliferation for the cytotoxic effect, through DNA chain termination mediated by the anti-metabolites produced.[31] We evaluated whether the

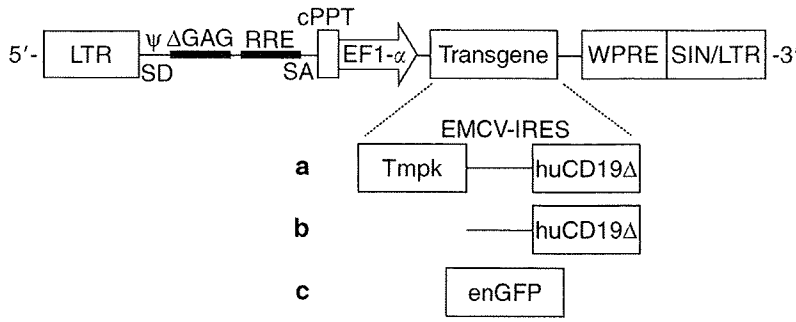

Figure 1 Schematic diagram of recombinant lentiviral vector (LV) constructs used in these studies. (**a**) LV-thymidylate kinase-IRES-huCD19Δ, (**b**) LV-IRES-huCD19Δ, (**c**) LV-enhanced green fluorescent protein. cPPT, central polypurine tract; EF-1α, elongation factor 1α promoter; LTR, long terminal repeat; RRE, Rev responsive element; SA, 3′ splice acceptor site; SD, 5′ splice donor site; SIN, self-inactivating LTR; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; ψ, human immunodeficiency virus packaging signal.

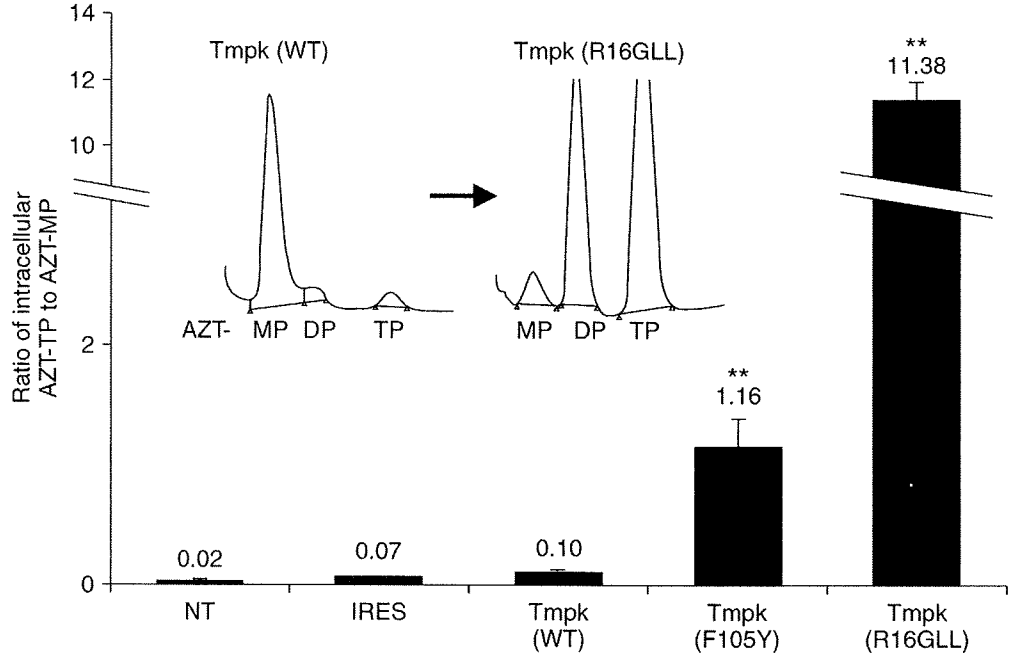

Figure 2 Determination of 3′-azido-3′-deoxythymidine (AZT) metabolites in transduced clonal Jurkat cell lines and controls treated for 36 hours with 100 μM AZT. Inset: representative chromatograms for the non-transduced cells and the thymidylate kinase R16GLL mutant–expressing cells. Peaks corresponding to AZT-monophosphate, AZT-diphosphate, and AZT-triphosphate are labeled. Bar graph: comparison of the ratio of the intracellular AZT-TP to AZT-MP in the AZT-treated cells. Data are mean ± standard error of the mean ($n = 3$). The statistical differences were evaluated by one-way analysis of variance followed by a Bonferroni post hoc test with the level of significance set at $P < 0.05$. $^{**}P < 0.01$ versus wild-type transduced cells.

cytotoxic events of AZT on tmpk-expressing cells were also dependent on cellular proliferation. We cultured transduced cells with or without 100 μM AZT in the presence of indirubin-3′-monoxime to arrest cell cycle progression. After incubation for 4 days with 5 μM indirubin-3′-monoxime, cells were arrested at the G_2/M-phase (data not shown). By treating the cells with 100 μM AZT in the presence of 5 μM indirubin-3′-monoxime, the apoptotic indices of the F105Y- and R16GLL-expressing cells were still significantly increased (2.3 ± 0.4-fold and 2.2 ± 0.2-fold, respectively) compared with the indices of cells without AZT treatment ($P = 0.0011$). No significant increases were seen in the apoptotic indices of control cells (data not shown). This suggests that the induction of apoptosis by AZT in the tmpk mutant–expressing cells is, in part, independent of their proliferation status.

Transduction and AZT sensitivity of primary human and mouse T cells

Next, primary human and mouse T cells were transduced once with the LV-tmpk constructs (multiplicity of infection = 20). We did not use the R16GLL mutant as this version contains a bacterial tmpk sequence, which could eventually be immunogenic *in vivo*. In contrast, the F105Y variant contains a single amino acid change at position 105. After 6 days of culture, transduced T cells were assessed for huCD19Δ expression. Although nominal NT cells expressed huCD19Δ, more than 50% of LV-transduced primary murine T cells stably expressed the marker (data not shown). In the same way, more than 60% of transduced human T cells stably expressed huCD19Δ (data not shown). These percentages are considerable given that expression of downstream genes in

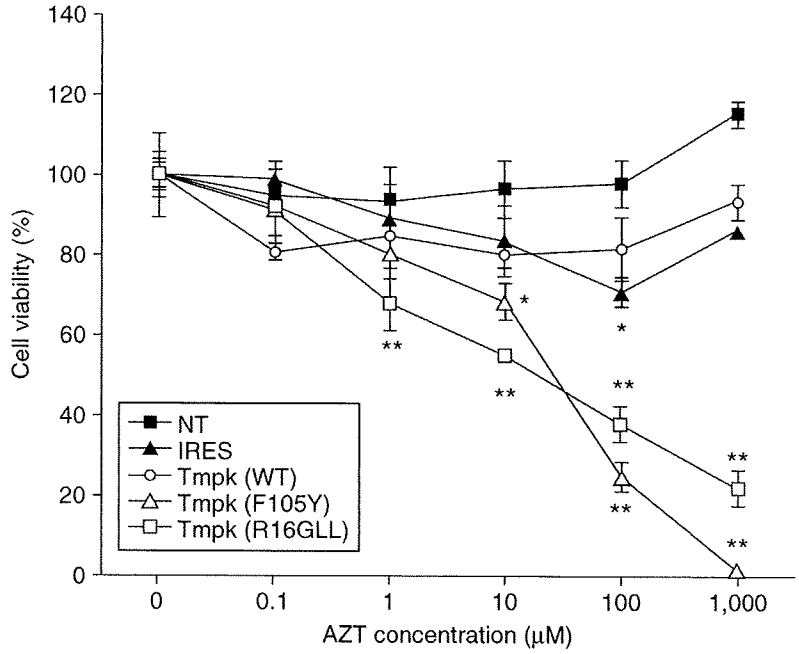

Figure 3 Measurement of 3′-azido-3′-deoxythymidine (AZT) sensitivity of clonally-derived Jurkat cells transduced with thymidylate kinase-IRES-huCD19Δ and control lentiviral vectors. Cell viability was measured by proliferation assay following 4 days incubation with or without AZT. The results were determined as a percentage of the A595 nm value from the assay. The negative control values (without AZT) and the values without cells were deemed to be 100 and 0%, respectively. Data are presented as mean ± standard error of the mean ($n = 3$). The statistical significance of experimental observation was determined by one-way analysis of variance followed by a Dunnett post hoc test with the level of significance set at $P < 0.05$ compared with the values of the control group of cells that were not treated with AZT. *$P < 0.05$ and **$P < 0.01$ versus cells without AZT treatment in each group.

bi-cistronic cassettes may be less than 10% that of upstream genes.[32] To test AZT sensitivity, transduced human T cells were exposed to 100 μM AZT for 4 days and induction of apoptosis was measured by Annexin V staining. Although the early apoptotic indices of primary NT human T cells were somewhat increased by AZT exposure at this dose, the apoptotic index of cells expressing the F105Y tmpk mutant was significantly increased ($P < 0.0001$) compared to the index of cells without AZT treatment (4.0 ± 0.3-fold; data not shown).

Novel suicide mechanism utilized by the tmpk/AZT axis

AZT inhibits HIV replication. Yet HIV-AIDS patients treated with AZT sometimes develop toxic mitochondrial myopathy through induction of mitochondrial biochemical dysfunction.[33–35] We sought to determine whether cellular apoptosis induction in our study involved this mechanism. We measured the mitochondrial inner membrane potential in intact LV-tmpk mutant–transduced cells following AZT treatment. Here we applied a fluorescent probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), and examined living cells by flow cytometry. The dye JC-1 emits a green fluorescence at low mitochondrial membrane potential.[36] At higher membrane potentials, JC-1 forms red fluorescence-emitting "J-aggregates." We found that a significant increase ($P < 0.0001$) in the loss of mitochondrial inner membrane potential occurred in both the F105Y- and the R16GLL-expressing Jurkat cells (**Figure 4a**) after 4 days of AZT treatment compared with controls. This effect was clearly present in the variant tmpk-expressing Jurkat cells but not in the WT-overexpressing cells from day 1. Indeed, control groups treated with AZT did not demonstrate a similar loss of membrane potential at any point (**Figure 4a**).

Caspase-3 is a key molecule in the cellular apoptosis pathway; loss of mitochondrial inner membrane potential induces caspase-3 activation.[37] We next evaluated caspase-3 activation in transduced cells treated with AZT. F105Y- or R16GLL-expressing cells treated with AZT showed a significant increase in the percentage of activated caspase-3-positive cells compared with untreated or control cells (**Figure 4b**). Interestingly, tmpk WT–overexpressing cells treated with AZT showed a slight, but significant, increase in the percentage of active caspase-3-positive cells. Taken together, our data demonstrate that apoptosis induction by prodrug in the tmpk mutant–expressing cells is due to the activation of caspase-3 resulting from the increase in the loss of the mitochondrial membrane potential caused by the accumulation of AZT-TP.

In vivo killing of LV/tmpk-transduced cells mediated by AZT

We next examined killing of the LV/tmpk mutant–transduced cells in an *in vivo* tumor model. K562 erythroid leukemia cells were transduced with our recombinant LVs. Since transduction with the F105Y LV was modest (~68% huCD19Δ-positive cells; data not shown), these cells were enriched by fluorescence-activated cell sorting (FACS) using anti-human CD19 conjugated to phycoerythrin. Afterward, more than 95% of cells were huCD19Δ-positive (data not shown), confirming the auxiliary utility of this marker for immuno-affinity enrichment. Minimal differences in growth characteristics of the transduced cells

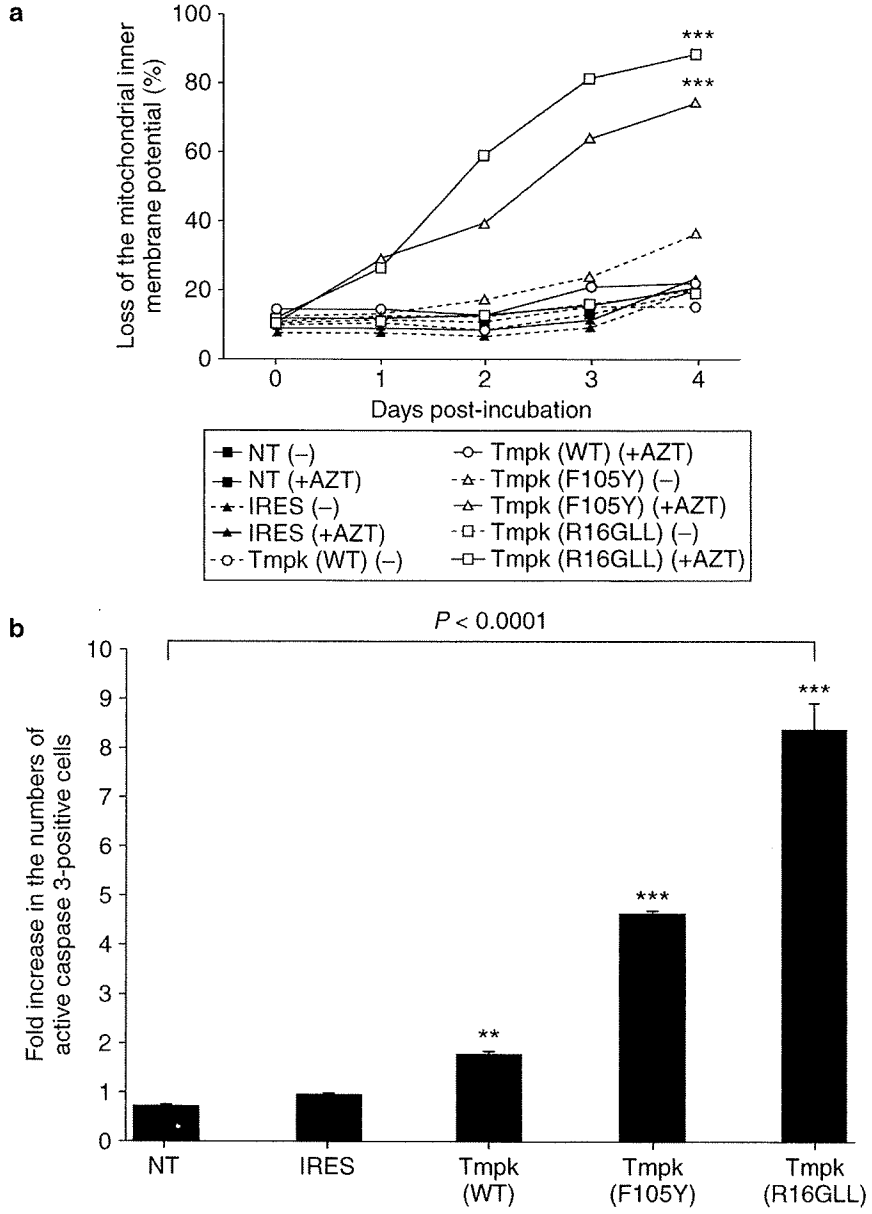

Figure 4 Analysis of the mechanism of induction of apoptosis by 3′-azido-3′-deoxythymidine (AZT) in the thymidylate kinase (tmpk) mutant–expressing cells. (**a**) The tmpk mutant–expressing cells treated with AZT showed an increase in the loss of mitochondrial membrane potential. Following 4 days incubation with or without 100 μM AZT, cells were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide for 15 minutes at 37 °C and were then analyzed by flow cytometry. To determine the effect of AZT on the increase in the loss of mitochondrial membrane potential at Day 4, the statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test with the level of significance set at $P < 0.05$. ***$P < 0.001$ ($n = 3$). (**b**) Activation of caspase-3 in transduced cells by AZT treatment. Cells were cultured for 4 days with or without 100 μM AZT. To compare the effect of AZT on activation of caspase-3 in each group, measurements of flow cytometry obtained from the cells treated with AZT were normalized by dividing by the values for cells without AZT. Data are mean ± standard error of the mean ($n = 3$). The statistical differences were evaluated by one-way ANOVA followed by a Bonferroni post hoc test with the level of significance set at $P < 0.05$. **$P < 0.01$ and ***$P < 0.001$ versus non-transduced cells.

were observed (data not shown). Next, 2×10^7 transduced K562 cells were injected into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Starting 1 day after the cell injection, the mice received daily injections of AZT (2.5 mg/kg/day) or vehicle for 2 weeks. Mice were killed when tumors reached approximately 1.5 cm^3; in animals injected with NT K562 cells, this endpoint occurred within 2 weeks. Mice not receiving AZT treatment quickly developed large tumors in a time-dependent manner (**Figure 5**). In contrast, the growth of K562 cells transduced with either of the tmpk mutant LVs (F105Y or R16GLL) was strongly inhibited by daily AZT injection, and the effects were sustained over time (**Figure 5**). This result, using this low dose of 2.5 mg/kg/day of AZT, is even more striking given safety data for mice treated with AZT at 270 mg/kg/day for 15 days, which showed only marginal hematoxicity and no nephrotoxicity.[38] This is also well below the Food and Drug Administration recommended dose of 1,200 mg/day acutely (17.1 mg/kg/day for a 70 kg individual) and 500–600 mg/day

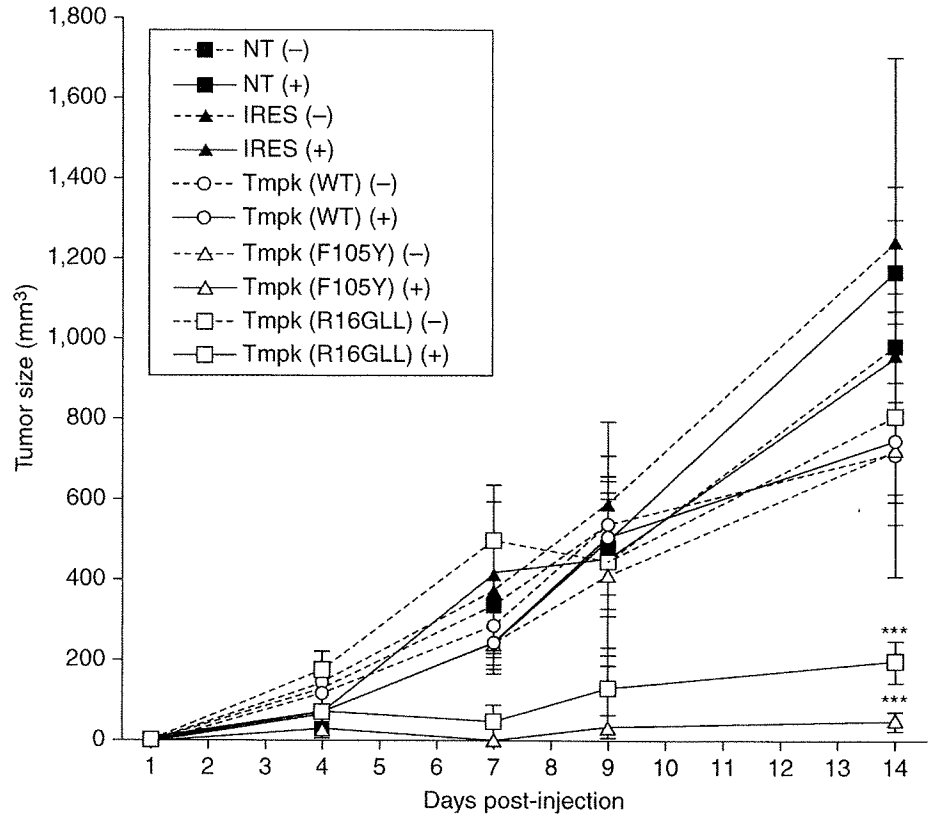

Figure 5 Daily injection of 3′-azido-3′-deoxythymidine (AZT) prevents growth of K562 cells transduced with lentiviral vector (LV)-thymidylate kinase mutant in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. NOD/SCID mice were subcutaneously injected into the dorsal right flank with 2×10^7 of either the non-transduced (NT) or the LV-transduced K562 cells. Starting 1 day after the cell injection, the mice received daily intraperitoneal injections of AZT (2.5 mg/kg/day) for 2 weeks and tumor volume was monitored. Data are mean ± standard error of the mean ($n = 5$ for each group). Statistical differences in tumor sizes at Day 14 were evaluated by one-way analysis of variance followed by a Bonferroni post hoc test with the level of significance set at $P < 0.05$. ***$P < 0.001$ versus NT without AZT injection.

(7.1–8.6 mg/kg/day for a 70 kg individual) for long-term treatment of HIV infection in humans.

DISCUSSION

Suicide gene therapy has a number of manifestations: direct tumor reduction, clearance of transplanted cells, and as a future safety component in clinical protocols involving integrating viral vectors. Such a portable safety system could also have benefits in stem cell transplantation.[2] Yet, to date, suicide gene therapy has not been fully effective. Here we have shown that overexpression of rationally designed mutant forms of human tmpk with improved kinetics significantly reduces cellular viability following AZT treatment both *in vitro* and *in vivo*. Thus, an otherwise minimally cytotoxic prodrug can be made highly cytotoxic by virtue of accelerating its activation. Overexpression of the WT enzyme alone was not sufficient; engineered enzymes with improved kinetics are required. Our results also demonstrate that the mechanism of AZT-induced apoptosis is associated with loss of mitochondrial inner membrane potential and activation of caspase-3 in the tmpk mutant–expressing cells. This mechanism differs from most previous suicide schemas and is likely beneficial, as it allows killing of non-dividing cells.

Tmpk catalyzes the rate-limiting second phosphorylation step in the activation of AZT.[14] AZT was the first effective treatment for AIDS patients;[11,12] however, long-term use can induce a severe myopathy characterized by alterations in mitochondria as a result of accumulation of AZT-MP.[34,39,40] Inhibition of the mitochondrial inner membrane potential has also been found in long-term AZT-treated rats.[35] We observed that accumulation of AZT-TP in the tmpk mutant–expressing cells abolished the inner membrane potential of mitochondria (**Figure 4a**) and increased the apoptotic index as a result of the activation of caspase-3 (**Figure 4b**). Yet the mechanism of this inhibition remains to be addressed in detail.

The use of huCD19Δ as a cell-surface marker facilitated an increase in the ratio of gene-modified cells by immuno-affinity enrichment. The contribution of the CD19 cytoplasmic domain to signal transduction has been previously assessed *in vitro* by transfecting the cells with a truncated form,[41] and *in vivo* using mice that express truncated human CD19.[30] These studies demonstrated that the cytoplasmic domain of CD19 is crucial for signaling and for the *in vivo* function of the CD19/CD21/CD81/Leu-13 complex. This indicates that the huCD19Δ form that we have employed is unlikely to signal. However, the effect of expression of huCD19Δ in target cells of interest warrants further examination. Of note, we have recently shown that use of a co-expressed surface antigen from bi-cistronic LVs allows clearance of transduced cells by other methods such as specific toxin-conjugated antibodies, thereby providing a further safety mechanism (J.A.M, manuscript submitted).

We have demonstrated that safety issues for integrating gene transfer can be alleviated using recombinant LVs that lead to expression of rationally engineered activating enzymes. Furthermore, NOD/SCID mice xenografted with transduced K562 cells (either F105Y or RG16LL expressing) and treated with AZT showed substantive suppression of tumor growth *in vivo* (**Figure 5**). Indeed, the transduced cancer cells are effectively eliminated at low systemic concentrations of AZT. This strategy can prevent cancer-related complications of gene therapy, as our killing schema involving the mitochondrial death pathway is robust enough that even immortalized cancer cells die by this mechanism. This could also lead to novel strategies in cancer treatment to overcome drug resistance that stems from mutations in cellular enzymes adversely affecting prodrug activation.

Adoptive immunotherapy using T cells is an effective approach to treat hematological malignancies.[9,42–45] Graft-versus-host disease, however, remains a major problem following non-T-cell-depleted allogeneic bone marrow transplantation.[46] An efficient *in vivo* safety switch for elimination of gene-modified T cells in the event of graft-versus-host disease would be useful. GCV has been used to deplete HSV-tk-expressing allogeneic lymphocytes following bone marrow transplantation.[42,43] Depletion is not always complete, however, and host immune responses against cells expressing this foreign enzyme can impair their function and persistence.[8,9] In addition, T-cell responses are to multiple epitopes, suggesting that modification of immunogenic sequences in HSV-tk would be ineffective in ablating this full response.[9] The use of human proteins as alternative suicide effectors is less likely to induce an immune response. Furthermore, most bone marrow transplantation patients are on prophylactic GCV to minimize cytomegalovirus infections, which decreases the clinical utility of HSV-tk-based suicide gene therapy. One of our current efforts seeks to utilize the novel LV-tmpk approach we have developed for reduction of graft-versus-host disease. Indeed, to confirm the utility of catalytically improved variants of human tmpk as an *in vivo* safety switch, we have already transduced primary cultured human and murine T cells in high transduction efficiencies and demonstrated specific killing upon addition of AZT (see above).

In conclusion, suicide gene therapy using LV-mediated human tmpk gene delivery combined with AZT as an effective prodrug has potential to be a potent safety element in controlling the fate of gene-modified cells.

MATERIALS AND METHODS

cDNA cloning of human CD19 and construction of LV shuttle vector. Total RNA was extracted from the human Burkitt's lymphoma cell line (Raji) using the TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA templates were generated from total RNA by reverse transcription using oligo-dT primer and Superscript II reverse transcriptase (Invitrogen). The cDNA of full-length huCD19 was obtained by PCR using platinum Hifi *Taq* DNA polymerase (Invitrogen) and primers CD19 F1 and CD19 R1 (described below). The amplified PCR product was directly ligated into the TA vector, pPCR-script SK (+) (Stratagene, La Jolla, CA), to give pPCR-huCD19full. A truncated form of huCD19 (huCD19Δ) that has the extracellular and transmembrane domains but lacks the cytoplasmic domain was generated by inverse PCR from pPCR-huCD19full using primers CD19 F2 and CD19 R2 (described below) to give pPCR-huCD19Δ. The F2 primer has a complementary sequence to the stop codon just after the end of the transmembrane domain. Following sequence confirmation of the cDNA inserts in pPCR-huCD19Δ, the cDNA fragments were then isolated and subcloned into the *Eco*RI site of the shuttle vector pSV-IRES, which has a sequence for an IRES element from the encephalo-myocarditis virus, to give pSV-IRES-huCD19Δ. The primer sequences used for subcloning of the human CD19 cDNA were as follows: CD19 F1: 5′-atgccacctcctcgcctcctcttcttcc-3′ and CD19 R1: 5′-tcacctggtgctccaggtgccc-3′. The truncated CD19 construct was made by inverse PCR using the following primers: CD19 F2: 5′-ccgccaccgcg gtggagctccag-3′ and CD19 R2: 5′-ttaaagatgaagaatgcccacaaggg-3′.

Subcloning of human tmpk cDNA and construction of bi-cistronic LVs. To subclone the cDNA for WT human tmpk, peripheral blood mononuclear cells were isolated from heparinized blood obtained from healthy donors by Ficoll-Hypaque density gradient separations (GE Healthcare Biosciences, Inc., Freiburg, Germany). The WT human tmpk cDNA was amplified by PCR using first-strand cDNA generated from peripheral blood mononuclear cell RNA by the method above. PCR products containing the WT tmpk cDNA were subcloned into pPCR-scriptSK (+) and sequenced. Mutant forms of tmpk, denoted F105Y and R16GLL, had been generated previously.[15,16] The cDNAs for the WT and each mutant form of tmpk were first subcloned into a shuttle vector (pSV-IRES-huCD19Δ) to construct bi-cistronic expression cassettes that allow simultaneous expression of a single mRNA strand encoding the suicide gene and huCD19Δ. Each of the constructs was then subcloned downstream of the internal EF1α promoter into an HIV-1-based recombinant LV plasmid, pHR′-cPPT-EF-W-SIN.[18] As a control for the transduction experiments, we constructed a pHR′-cPPT-EF-IRES-huCD19Δ-W-SIN LV by subcloning the IRES-huCD19Δ cassette from the pSV-IRES-huCD19Δ plasmid into pHR′-cPPT-EF-W-SIN. In addition, we used the pHR′-cPPT-EF-enGFP-W-SIN LV[24] containing the enhanced GFP cDNA.

Preparation of high-titer LV. Vesicular stomatitis virus glycoprotein–pseudotyped LVs, including an enhanced GFP-marking vector, were generated by transient transfection of 293T cells with a three-plasmid system (the aforementioned pHR′ plasmid constructs, the packaging plasmid pCMVΔR8.91, and the vesicular stomatitis virus glycoprotein envelope encoding plasmid pMD.G) using $CaPO_4$ precipitation.[17] Viral supernatants were harvested 48 hours later, passed through a 0.45-μm filter, and suspended in phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin after ultracentrifugation at 50,000*g* for 2 hours at 4 °C. The concentrated viral supernatants were serially diluted and titered on 293T cells. Transgene expression in transduced cells was assessed 72 hours later using a FACS Calibur (BD Biosciences, San Jose, CA) after staining of the transduced and control cells with monoclonal mouse anti-human CD19 conjugated with phycoerythrin (BD Biosciences, San Jose, CA) or for enhanced GFP expression. Analysis of the data was performed using Cell Quest software (BD Biosciences). Titers of each of the concentrated LVs used were more than 10^8 productively infectious particles/mL as measured by functional enhanced GFP and huCD19Δ expression (data not shown).

Transduction and analysis of transgene expression by western blot and flow cytometric analyses. Cells of the human T lymphoma cell line, Jurkat, and of the human erythro-leukemic cell line, K562, were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (CPAA Laboratories, Etobicoke, ON), 100 U/ml penicillin, and streptomycin to 100 μg/ml (both Sigma, Oakville, ON). Cells were infected with concentrated virus stocks using a multiplicity of infection of 10 in the presence of 8 μg/ml protamine sulfate. Infected cells were then kept in culture for 5 days before evaluating gene transfer efficiency. Gene transfer efficiencies were measured by

flow cytometry as described above. Individual clone cell lines were used for all subsequent experiments. They were derived by limiting dilution and selected based on comparable huCD19Δ expression as determined by flow cytometry (above).

To compare the relative expression levels of tmpk by flow cytometry, the transduced cells were first fixed with 4% buffered formalin for 15 minutes and then permeabilized by treatment with phosphate-buffered saline containing 0.1% Triton X-100 for 10 minutes. Cells were incubated with 20% normal goat serum for 30 minutes and then incubated with rabbit anti-human tmpk (diluted 1:500) for 1 hour. The cells were further incubated with goat anti-rabbit IgG conjugated to Alexa488 (diluted 1:500, Molecular Probes Inc., Eugene, OR) for 1 hour. All incubations were performed at room temperature. For western blots, total cell lysates were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride filters (Millipore, Billerica, MA). Filters were blocked with 5% fat-free skim milk in tris-buffered saline with 0.05% Tween-20. Human tmpk overexpression was elucidated using rabbit anti-human tmpk (diluted 1:5,000). Lysate protein loading amounts in each lane were confirmed with a murine anti-β-actin antibody (Chemicon, Temeula, CA) diluted 1:5,000. Blots were probed with appropriate horseradish peroxidase–conjugated secondary antibodies and proteins were detected using an enhanced chemiluminescence kit (Perkin Elmer, Norwalk, CT) and Kodak BioMAX XAR film.

HPLC for AZT metabolites. Cells were cultured in the presence of 100 μM AZT for 36 hours. A total of 10^7 cells were homogenized by sonication in 100 μl of 5% (w/v) trichloroacetic acid. The supernatant was collected after centrifugation at 10,000 *g* for 15 minutes at 4 °C. Trichloroacetic acid was removed by extraction with an equal volume of 20% *tri*-n-octylamine in pentane. The neutralized aqueous fraction was directly injected into the HPLC machine (Waters, Milford, MA). Separation of AZT and its metabolites was performed on a C18 column (Waters), with a mobile phase composed of 0.2 M phosphate buffer containing 4 mM tetrabutylammonium hydrogen sulfate (pH 7.5) and acetonitrile in the ratio of 97:3 (v/v)[47] at a flow rate of 1.5 ml/min. The UV absorbance was monitored at 270 nm. Standards for each AZT metabolite (AZT-MP, AZT-DP, and AZT-TP) were purchased from Moravek Biochemicals (Brea, CA). Five million cell equivalents were injected and analyzed in triplicate. Mitochondria-enriched fractions from 10^9 cells of LV-IRES-huCD19Δ- and LV-R16GLLtmpk-IRES-huCD19Δ-transduced clones treated with 100 μM AZT were prepared using a method described elsewhere (AT Ho and E Zacksenhaus, manuscript submitted). Specifically, cells were lysed mechanically in a glass grinder in 5 ml of ice-cold S100 buffer (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na·EDTA, 1 mM Na·EGTA, 1 mM DTT, and 0.1 mM PMSF). Lysate was then centrifuged at 700 g for 20 minutes at 4 °C; supernatant was collected and centrifuged further at 10,000 *g* for 30 minutes at 4 °C. Next the supernatant was collected as the cytosol fraction, and the mitochondria-enriched pellet was washed with 1 volume of ice-cold S100 buffer, followed by centrifugation at 10,000 *g* for 30 minutes at 4 °C. AZT metabolites were extracted from the pellet as before and injected into the HPLC in duplicate.

Determination of AZT sensitivity of tmpk-transduced Jurkat cells. Transduced Jurkat cells and single-cell clones were seeded in 96-well plates (2×10^5 cells/well) in 200 μl of the Roswell Park Memorial Institute medium described above, with increasing concentrations of AZT (0, 0.1, 1, 10,100 μM, and 1 mM). The medium was changed daily. After 4 days of culture, cell viability was determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI).

For evaluation of the induction of apoptosis, treated Jurkat clonal cells were stained with Annexin V. In brief, cells were seeded in a 24-well plate (10^6 cells/well) in 1 ml medium with or without 100 μM AZT. After 4 days of culture, Annexin V staining was performed according to the manufacturer's protocol (Annexin V-APC; BD Pharmingen, San Diego, CA). To test whether AZT-mediated cell killing depends on the cellular proliferation, indirubin-3′-monoxime (final concentration 5 μM; Sigma-Aldrich, St. Louis, MO) was added simultaneously with 100 μM AZT to the culture. To simplify comparative studies, a relative apoptotic index was calculated. Here data obtained were normalized by dividing results from AZT-treated cells in each condition by the results obtained without added AZT. Values were reported as fold increases. Statistical significance between groups was calculated by analysis of variance.

Transduction of primary T cells with LVs and evaluation of induction of apoptosis after AZT exposure. Human T lymphocytes were isolated from peripheral blood mononuclear cells contained within heparinized blood obtained from healthy human donors by Ficoll-Hypaque separations. Mouse T cells were prepared from B-cell-depleted splenocyte preparations using goat anti-mouse IgG beads (BioMag, Qiagen, Mississauga, ON). T cells were activated using anti-CD3- and anti-CD28-coated beads[48] in a ratio of 1:3 (cells to beads) with 20 IU/ml recombinant human interleukin-2 (R&D Systems, Minneapolis, MN) for 3 days. Cells were infected with concentrated virus stocks for 3 hours on ice using an indicated multiplicity of infection in the presence of 8 μg/ml protamine sulfate. Infected cells were then kept in culture for 5 days before gene transfer efficiency was evaluated. Gene transfer efficiencies were measured by flow cytometry using a monoclonal anti-human CD19 antibody conjugated with phycoerythrin as described above. Induction of apoptosis following AZT exposure was evaluated by Annexin V staining as above.

Measurement of mitochondrial inner membrane potential and activation of caspase-3. Transduced cells (10^6) were treated with 100 μM AZT for 4 days or left untreated. To detect changes in the mitochondrial inner membrane potential, the cells were incubated with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes Inc.) for 30 minutes at 37 °C and were then analyzed using a FACS Calibur. The activation of caspase-3 in cells was examined using the FACS Calibur after incubation with a fluoroscence isothiocyanate–labeled caspase-3 inhibitor peptide (FITC-DEVD-FMK, Calbiochem, San Diego, CA) for 1 hour at 37 °C.

Transduced K562 cells in a NOD/SCID xenograft model. Transduced K562 cells were affinity-purified by MACS using magnetic beads conjugated with an anti-human CD19 monoclonal antibody (Miltenyi Biotec Inc., Auburn, CA) or by FACS. The purity of the cells following isolation was evaluated using the FACS Calibur. NOD/SCID mice (5–8 weeks old, purchased from Jackson Laboratories, Bar Harbor, ME) were maintained at the Animal Resource Centre at the Princess Margaret Hospital (Toronto, ON, Canada). The entire animal experimental procedure followed a protocol approved by the Animal Care Committee of the University Health Network. Experimental groups consisted of male and female NOD/SCID mice injected with 2×10^7 K562 cells (re-suspended in 0.5 mL D-phosphate-buffered saline, Oxoid, Basingstoke, England) that were either lentivirally transduced ($n = 10$ animals for each LV construct) or NT ($n = 10$). Injections were performed subcutaneously into the dorsal right flanks of recipient mice as previously described.[49] One day after injection of the cells, half of the mice in each group ($n = 5$) began receiving daily AZT injections, administered intraperitoneally at a dose of 2.5 mg/kg/day for 14 days. Tumor growth was measured by caliper and calculated as $0.5 \times \text{length} \times \text{width}^2$ (in mm^3) for up to 14 days after inoculations.

Statistical analysis. Data are presented as the mean ± standard error of the mean for *in vitro* experiments and the mean ± standard deviation of the mean for *in vivo* experiments. Statistical analyses were performed using StatView version 4.5 software for Macintosh (SAS, Cary, NC). For *in vitro* experiments, a one-way analysis of variance with either a Bonferroni or a

Dunnett post hoc test was used to determine statistically significant results, with the level of significance set at $P < 0.05$. Statistical comparison of means was performed using a two-tailed unpaired Student's t-test for *in vivo* experiments.

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