対応する1種類のプライマーを用いて、アダプター付加DNAフラグメントをPCR増幅した。200～1100bp サイズのフラグメントを優先的に増幅するようにPCRを行い、増幅産物を断片化し、Terminal Deoxynucleotidyl Transferaseにて末端Biotin標識した。標識したDNAはGeneChip Mapping 500K Setアレイにハイブリダイゼーション後（Hybridization Oven 使用），専用装置Fluidics Station（GeneChip Fluidics Station 450）を用いて洗浄およびstreptavidin-phycoerythrinの染色を行い、レーザースキャナー(GeneChip Scanner 3000)でデータ収集を行った。

5）分化能の測定

脂肪細胞への分化能を測定するため、カバースリップの上に培養した各細胞を誘導培地（hMSC Differentiation BulletKit -Adipogenic；PT-3004, Camblex BioScience Waltervillo, Inc. USA）、神経細胞への誘導にはNPMM Bullet kit (NPMM™ BulletKit (B3209, Camblex BioScience Waltervillo, Inc. USA) を用いた。骨芽脂肪への分化誘導には0.1 μM dexamethasone (Sigma Chemical Co., USA), 50 μg/ml L-ascorbic acid (Sigma Chemical Co., USA) と10 mM β-glycerophosphate (Sigma Chemical Co., USA) をPlusoid-M 培地 (Med-Shirotori Co., Tokyo, Japan) 培地またはPoweredby10 培地 (Med-Shirotori Co., Tokyo, Japan) に入れ、2-4 週間培養した。phosphate-buffered saline (PBS)で洗浄後，4% paraformaldehydeで固定した。

脂肪細胞はOil Red-O (Sigma Chemical Co., USA)染色し、骨芽細胞には0.25 mg/ml naphthol AS-BI phosphate および0.25 mg/ml Fast violet LB salt でalkaline phosphatase染色した。神経細胞の観察には、パラフォルムアルデヒドとメタノール固定したのち、anti-Ⅲβ tubulin 抗体 (Sigma Chemical Co. USA) またはanti -neurofilament antibody NF-200 (Sigma Chemical Co., USA) とTexas Red-anti -mouse IgG 抗体 (Southern Biotechnology Associates, Inc., USA) で免疫染色した。

C. 研究結果

1）アレイCGHの感度・再現性確認

研究に用いるアレイ CGH (Human Genome CGH 244A) の感度・再現性を確認するために正常細胞から抽出したDNAとがん細胞から抽出したDNAを用いて解析を実施した。感度として、正常DNAに対してがんDNAを混合して解析することにより、3 割程度の混合において十分に2つのサンプルにおけるゲノムの増減を区別できる感度を有していること、ならびに、10kb程度の欠失をシグナルとして検出できることが明らかとなった。さらにこれらの結果に関して再現性を確認したが、その感度に変化は見られなかった。

2）不死化間葉系幹細胞における染色体解析・ゲノム性合解析

長期培養を行った不死化間葉系幹細胞UE6E7T-3においては染色体数の変化がみられた。PDL 62のUE6E7T-3は90 %の細胞が46本の染色体をもっていたが、PDL 147では染色体欠失により44本になった細胞が43%観察された。

次に、染色体の不安定性を調べるために、アレイ CGH 法による解析を行ったところ13

番染色体1本と16番染色体の長腕部の欠失が確認された（図１）。この13番染色体１本の欠失に着目し、間期の細胞における13番染色体ならびに中心体の蛍光染色を行った。間期においても培養初期の細胞には13番染色体2本が観察され、長期培養後の細胞においては13番染色体が１本しか認められなかった。また、中心体の蛍光染色により欠失が起こる細胞において中心体の数の増加が認められること、ならびにそれに伴う13番染色体の不均等分割が起こることを明らかにした（図２）。欠失する 13 番染色体がランダムであるのか特定アレルであるのかを明らかにするため、SNPチップによる解析を実施した。13番染色体および16番染色体に長腕部におけるSNPタイピングの出現は欠失がランダムに起こる場合にはAA：AB：BB＝１：２：１の割合でタイピングされるはずであるが長期培養後の細胞にはABとタイピングされるSNPは殆ど存在しなかった。このことから欠失は特定アレルに起こることが明らかとなった（図３）。

３）細胞の分化能評価

間葉系幹細胞は骨芽細胞、軟骨細胞や脂肪細胞に分化することができるし、ときには神経様細胞に分化したりすることが可能であり、すなわち多分化能を持っていると報告されている。今回用いた間葉系幹細胞を適切な誘導培地で２～４週間培養した。特に、UE6E7T-3細胞は他の細胞よりも脂肪細胞へ分化する能力が強いことが明らかとなった。このことは染色体の変化が起こっても変化しないことを明らかとした。

３）ヒトES・iPS細胞のゲノム詳細解析

京都大学より2008年8月にヒトES細胞３株の分配を受け、９月より細胞の長期培養を開始した。長期培養に伴うゲノム変化の解析を実施しており、今後培養条件、細胞の継代方法、凍結方法など細胞培養に関わる基礎項目に関して条件設定を行い、その際の表現型とゲノム詳細解析結果との関係を解析する予定である。また、ヒトiPS細胞に関しても2008年10月に入手し培養を開始しており、ゲノム詳細解析を実施中である。プレリミナリーな結果としてヒトiPS細胞に染色体変化が起こっていること確認された。今後ヒトES細胞と同様に表現型とゲノム詳細解析結果との関係解析を行う予定である。

D. 考察

本研究では、アレイCGHの感度と再現性の確認を行った。アレイCGHによるコピーナンバーバリアント（CNV）解析やLOH解析は病態との関係性が非常多く、近年注目が注がれている。健常成人のゲノム解析を行うとタンパクの昨日変化が予測されるような変異が３００近くの遺伝子において見出される。その数はヒトゲノム中にあるタンパクコード遺伝子の１%以上にも及び、ゲノムのコピー数異常であるCNVも数多く検出されている。このことは健康に日々暮らしている私たちにおいても機能の軽重を問わず多数の遺伝子に変異が起きているものの、たまたま細胞や臓器、運動器や神経、さらに精神発達などにおいて日常の生活に支障が無い程度におさまっているに過ぎないことを示している。しかし、近年の解析結果から、アンドロゲン代謝酵素（UGT2B17）と前立腺がんの発症、セリンプロテアーゼ遺伝子（PRSS1）と家族性膵炎など病態（表現型）との関係が明らかにされる例も数多く存在している。本研究においてアレイ

CGH 解析を実施することにより細胞の詳細なゲノム解析を行い、細胞をキャラクタライズすることである。このキャラクタライズが実際には細胞の品質を評価するために表現型と結びつけることができれば非常に有用な方法となり、新たな評価方法となる。確認した感度は3割程度のゲノム量の変化を解析することが出来た。全ての細胞において変異が起こり、ゲノム量が変化した場合には2つあるアレルの1本分の増減に基づくことが考えられ、その増減量は5割と推定される。本研究の結果から十分に検出可能でありアレイ CGH が十分な感度であるとわかった。また、再現性に関しても同じ試料による解析によって同じ結果が得られることを確認できた。

遺伝子を導入したヒト骨髄間葉系幹細胞が *in vitro* 培養で遺伝子型、表現型にどのような変化を示すかを解析し明らかにした。本研究で使用した細胞株 UE6E7T-3 はヒトパピローマウイルス E6E7 遺伝子とｈTERT を用いて不死化されており長期培養を続けると、染色体数に大きな変化を示した。培養初期には diploid (2n)であるが、培養期間が長くなるにつれて、aneuploid(2n-1～2)、tetraploid (4n) と tetraploid より少し少ない異数体 aneuploid(4n-1～5)になるのが観察された。最近、ヒト N/TERT-1 ケラチノサイトや HeLa 細胞の in vitro 実験で、異数体 aneuploid が形成される前に tetraploid の形成があり、それは分裂期に２つの娘細胞の不分離によるという nondisjunction 説が報告されたが、古くからヒトがん細胞でも高頻度で tetraploid が観察されている。UE6E7T-3 において観察された aneuploid(2n-1～2)においては、17 番染色体は正常であるのに 13 番染色体の1本の欠失が UE6E7T-3 の 70～80%で観察された。このことから、細胞株が増殖していくためには 13 番染色体の欠失が重要であったことが示されている。特定染色体の欠失によるカリオタイプ変異はヒト ES 細胞でも報告されている。これまで染色体異数体は tetraploid から形成されるという主張が強かったが、本研究から tetraploid を経由せず、diploid からでも形成されるという異数体形成機構の新たな事実が示された。

tetraploid 形成なしに起こる異数体形成機構は不明であるが、中心体が重要な役割をしている可能性が高い。正常細胞では、中心体が細胞あたり 1～2 個しかないものが、本研究で使用したUE6E7T-3では3～10個も存在するものが細胞の 12～35%にも及ぶ。この中心体の増加が染色体の不均等分割につながったものと考えられる。

さらに本研究では SNP チップを用いて欠失する 13 番染色体がランダムに欠失するのか、あるいは特定のアレルが欠失するのかに関して解析を行った。その結果、特定アレルの欠失が起こっていることが証明され、そのメカニズムに非常に興味が持たれ、この細胞が異数体形成のメカニズムを解析するための良いモデルとなると考えられた。

細胞治療で最も重要な問題は移植する細胞の質的要素である。間葉系幹細胞が今日再生医療面で大きな期待を受けているのは、他の組織幹細胞ではみられないいろいろな他組織への分化能を持っているからである。この研究で用いた細胞株は臍帯血と骨髄由来の幹細胞であるが、*in vitro* の限られた条件下でも骨細胞、脂肪細胞、神経細胞への分化能を保持していた。遺伝子を導入してもその分化能を維持していた。さらに市販されているヒト間葉系幹細胞に関しても、長期培養における

染色体異常解析を行ったが、培地の違いによりその増殖速度に影響を与えることは明らかになったが、長期培養による染色体異常は観察されなかった。今後アレイCGHによる染色体のより詳細な解析を試み、より微細な染色体変化を、より簡便に解析する評価系開発を目指す。

E. 結論

細胞のゲノムDNAを用いて、アレイCGHの感度及び再現性を検証したが、染色体の増減を解析するのに十分な検出感度、ならびに再現性を示すことが明らかとなった。本研究に用いた遺伝子導入細胞株は13番染色体1本の特異的欠失を伴うが、間葉系細胞本来の分化能は保持していた。また、欠失する13番染色体が特定アレルに起こっていることが明らかとなり、この細胞株は異数体形成におけるモデル細胞として非常に有用であると考えられた。

今後、細胞治療がますます盛んになるが、ES細胞だけではなく、組織幹細胞でも移植に必要な細胞量の確保には *in vitro* 増幅が不可欠である。そのためには他細胞のコンタミネーションやマイコプラズマ汚染、ウイルス汚染チェックと同じように、移植された組織の悪性変異を防ぐためにもカリオタイプの検査は品質管理の重要な項目に加えなければならないことをこの研究は警告している。

F. 健康危険情報

適用なし。

G. 研究発表

1. 論文発表

(1) 水澤博, 小澤裕, 小原有弘, 増井徹, 佐藤元信, 岩瀬秀, 深海薫, 西條薫, 中村幸夫
培養細胞で頻発するクロスコンタミネーションへの警戒、実験医学;26(3):561-567 (2008)

(2) 水澤博, 小澤裕, 増井徹, 平田誠, 小原有弘
STR分析によるヒト培養細胞の迅速同定法ー培養細胞のクロスコンタミネーション防止のためにー、実験医学;26(9):1395-1403 (2008)

(3) Takeuchi M, Takeuchi K, Ozawa Y, Kohara A, Mizusawa H. Aneuploidy in immortalized human mesenchymal stem cells with non-random loss of chromosome 13 in culture. In Vitro Cell Dev Biol Anim (2009) in press.

2. 学会など口頭発表

① 小原有弘、水澤博
細胞バンクの現状と課題
日本組織培養学会第81回大会(つくば)

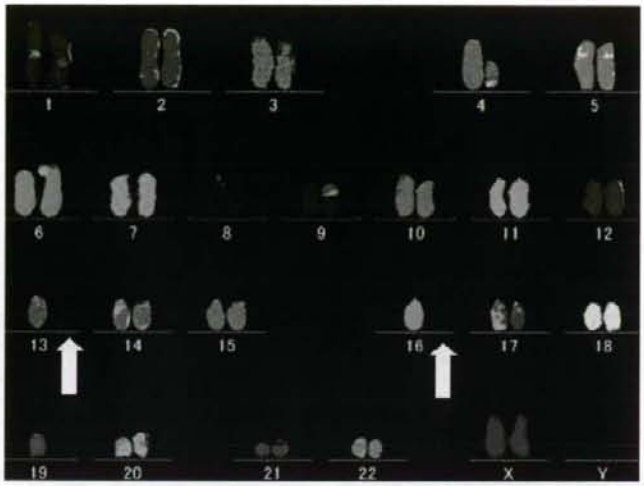
② Arihiro Kohara, Azusa Ohatani, Yutaka Ozawa, Setsuko Shioda, Tohru Masui, Hiroshi Mizusawa. Mycoplasma Contamination and Cross Contamination in Tissue Culture: A Survey of Major Institutions in Japan. Annual meeting of Symposium In Vitro Tecnology Jun. 2008 (Tucson CA).

③ Arihiro Kohara Genomic Stability Analysis of Immortalized Human Cells and Human Tumor Cells Using High-Resolution Array-Based

Comparative Genomic Hybridization. 48th ASCB Annual Meeting Dec. 2008.

④ Yutaka Ozawa, Masao Takeuchi, Kikuko Takeuchi, Arihiro Kohara, Hiroshi Mizusawa Gennomic Stability of Immortalized Human Mesenchymal Stem Cells (Non-random Loss of Chromosome 13 in Culture). 日本環境変異原学会第37回大会（沖縄2008年12月）

A



B

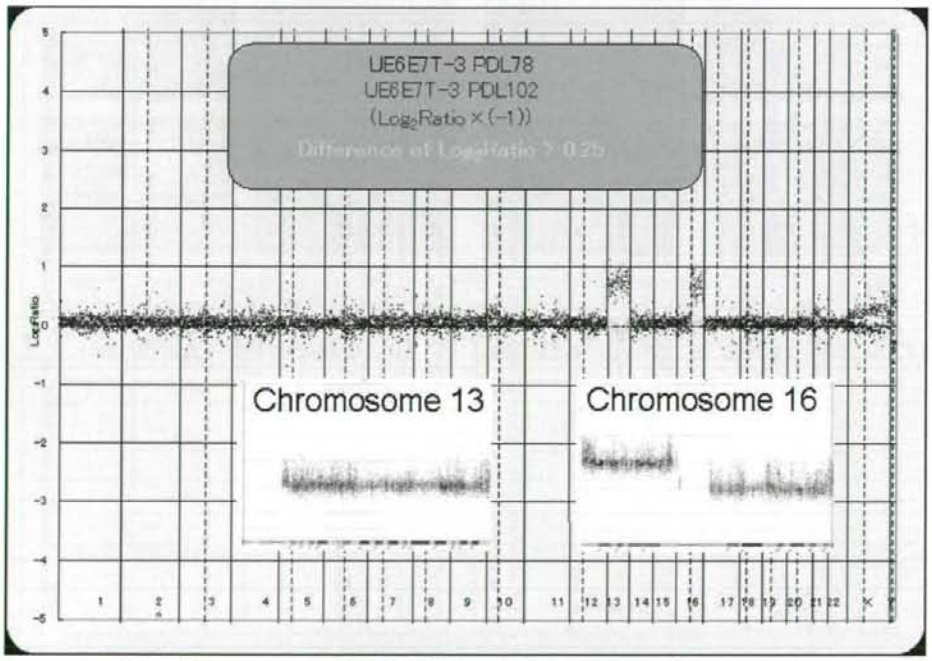


図1.　A：遺伝子導入したヒト間葉系幹細胞株UE6E7T-3のFISH 分析
UE6E7T-3 細胞では13 番染色体ならびに16 番染色体の1 コピーが欠失している。
B：遺伝子導入したヒト間葉系幹細胞株UE6E7T-3 のアレイCGH 解析
UE6E7T-3 細胞では13 番染色体ならびに16 番染色体の長腕部1 コピーが欠失している。

A

B

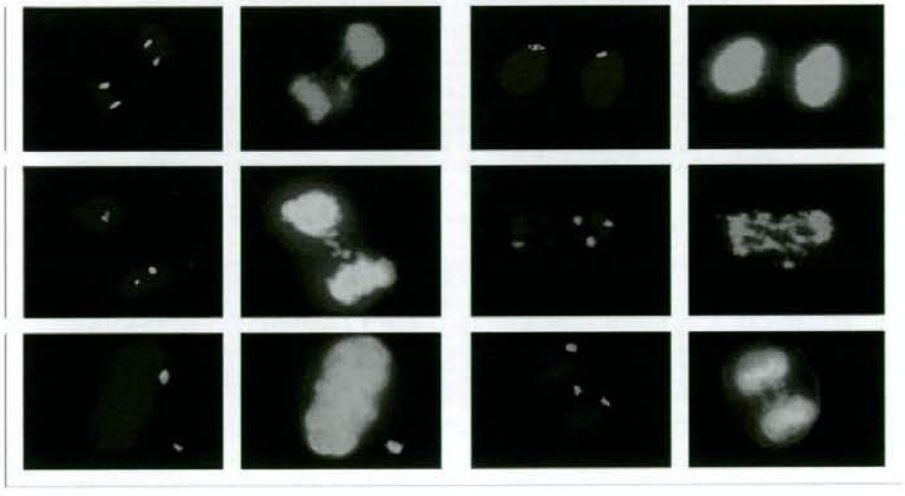


図２．UE6E7T-3 細胞における中心体の数の変化（A）と 13 番染色体の不均等分割（B）

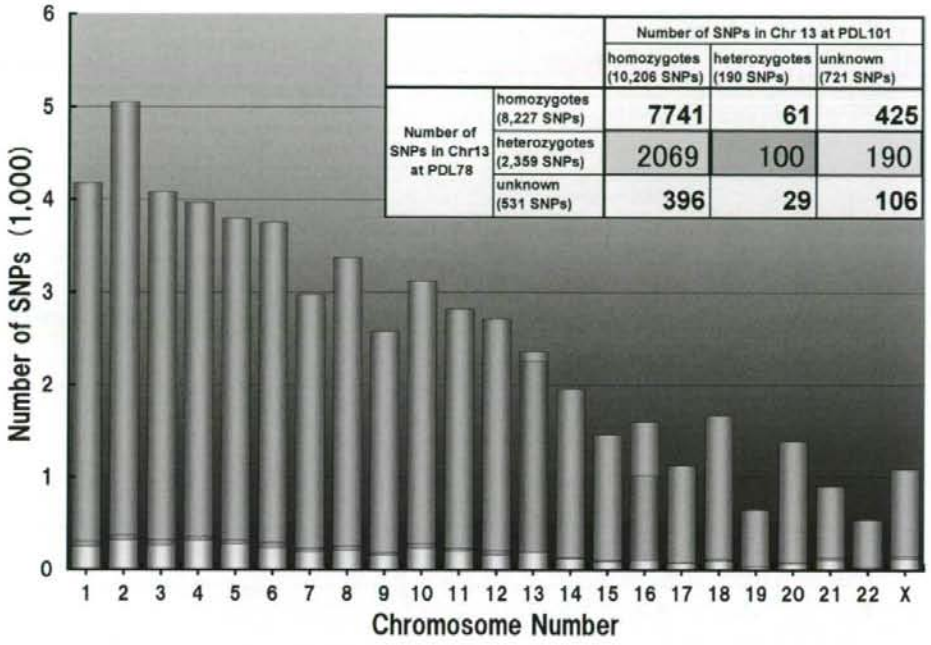


図3．欠失した13番染色体のSNPチップ解析

13番染色体および16番染色体の長腕部が欠失していることがアレイCGHで明らかとされており、その部分のSNPを解析すると、欠失する前の細胞においてヘテロであったSNPの大部分がホモであるとSNPチップの結果で示された。

別紙４

研究成果の刊行に関する一覧表レイアウト（参考）

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
Sakurai F, Nakamura SI, Akitomo K, Shibata H, Terao K, Kawabata K, Hayakawa T, Mizuguchi H.	Adenovirus serotype 35 vector-mediated transduction following direct administration into organs of nonhuman primates.	*Gene Ther.*	16	297-302	2009
Sakurai F, Nakamura S, Akitomo K, Shibata H, Terao K, Kawabata K, Hayakawa T, Mizuguchi H.	Transduction properties of adenovirus serotype 35 vectors after intravenous administration into nonhuman primates.	*Mol. Ther.*	16	726-733	2008
水澤博，小澤裕，小原有弘，増井徹，佐藤元信，岩瀬秀，深海薫，西條薫，中村幸夫	培養細胞で頻発するクロスコンタミネーションへの警戒	実験医学	26	561-567	2008
水澤博，小澤裕，増井徹，平田誠，小原有弘	STR分析によるヒト培養細胞の迅速同定法－培養細胞のクロスコンタミネーション防止のために－	実験医学	26	1395-1403	2008

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
Takeuchi M, Takeuchi K, Ozawa Y, Kohara A, Mizusawa H.	Aneuploidy in immortalized human mesenchymal stem cells with non-random loss of chromosome 13 in culture.	*In Vitro Cell Dev Biol Anim.*			印刷中

Gene Therapy (2009) 16, 297–302

www.nature.com/gt

SHORT COMMUNICATION

Adenovirus serotype 35 vector-mediated transduction following direct administration into organs of nonhuman primates

F Sakurai[1], S-i Nakamura[2,3,7], K Akitomo[1], H Shibata[2], K Terao[2], K Kawabata[1], T Hayakawa[4,5] and H Mizuguchi[1,6]

[1]*Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Ibaraki City, Osaka, Japan;* [2]*Tsukuba Primates Research Center, National Institute of Biomedical Innovation, Tsukuba City, Ibaraki, Japan;* [3]*The Corporation for Production and Research of Laboratory Primates, Tsukuba City, Ibaraki, Japan;* [4]*Pharmaceuticals and Medical Devices Agency, Chiyoda-Ku, Tokyo, Japan;* [5]*Pharmaceutical Research and Technology Institute, Kinki University, Osaka, Japan and* [6]*Graduate School of Pharmaceutical Sciences, Osaka University, Suita City, Osaka, Japan*

Adenovirus (Ad) serotype 35 (Ad35) vectors have attracted remarkable attention as alternatives to conventional Ad serotype 5 (Ad5) vectors. In a previous study, we showed that intravenously administered Ad35 vectors exhibited a safer profile than Ad5 vectors in cynomolgus monkeys, which ubiquitously express CD46, an Ad35 receptor, in a pattern similar to that in humans. However, the Ad35 vectors poorly transduced the organs. In this study, we examined the transduction properties of Ad35 vectors after local administration into organs of cynomolgus monkeys. The vectors transduced different types of cells depending on the organ. Hepatocytes and microglia were mainly transduced after the vectors were injected into the liver and cerebrum, respectively. Injection of the vectors into the femoral muscle resulted in the transduction of cells that appeared to be fibroblasts and/or macrophages. Conjunctival epithelial cells showed transgene expression following infusion into the vitreous body of the eyeball. Transgene expression was limited to areas around the injection points in most of the organs. In contrast, Ad35 vector-mediated transgene expression was not detected in any of the organs not injected with Ad35 vectors. These results suggest that Ad35 vectors are suitable for gene delivery by direct administration to organs.

Gene Therapy (2009) **16,** 297–302; doi:10.1038/gt.2008.154; published online 18 September 2008

Keywords: *adenovirus serotype 35 vector; local administration; nonhuman primate; CD46*

Adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses with icosahedral symmetry. To date, 51 human adenovirus (Ad) serotypes have been identified and classified into six species.[1,2] Among these serotypes, Ad serotype 5 (Ad5), which belongs to species C, is the basis of almost all the Ad vectors commonly used, including those used in clinical trials. Conventional Ad5 vectors have several advantages as gene delivery vehicles. However, it is now well established that the hurdles to Ad5 vector-mediated gene therapy are the high seroprevalence to Ad5 in adults and the refractoriness of cells lacking the expression of coxsackievirus-adenovirus receptor, which is a primary receptor for Ad5, to Ad5 vectors. Pre-existing anti-Ad5 immunity significantly decreases the transduction efficiencies of Ad5 vectors. Even when an Ad5 vector-based vaccine was administered locally into muscle, pre-existing anti-Ad5 antibodies reduced its efficacy.[3,4] A lack of coxsackievirus-adenovirus receptor expression renders the cells unsusceptible to Ad5 vectors at least *in vitro*. Important target cells for gene therapy, including hematopoietic stem cells and dendritic cells, often poorly express coxsackievirus-adenovirus receptor. In addition to these drawbacks, Ad5 vectors have high hepatic tropism. Even when Ad5 vectors are locally injected into a diseased area (for example, a tumor), they are drained from the injection sites into the systemic circulation and primarily transduce hepatocytes because of their high hepatic tropism; on the other hand, efficient transduction is obtained around the injection points. When Ad vectors carry a transgene that exerts cytotoxic effects on transduced cells, Ad vector-mediated hepatic transduction leads to severe hepatotoxicity.[5–7]

In contrast, human species B Ad serotype 35 (Ad35) vectors, which our group and several others have developed,[8–11] possess attractive properties that can overcome the drawbacks of conventional Ad5 vectors. First, Ad35 vector-mediated transduction is not hampered by anti-Ad5 antibodies, because Ad35 belongs to a different species (species B) than Ad5 (species C). Second, Ad35 vectors bind to human CD46 as a receptor.

Correspondence: Dr H Mizuguchi, Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Asagi, Saito, Ibaraki City, Osaka 567-0085, Japan.
E-mail: mizuguch@nibio.go.jp
[7]Current address: Research Center of Animal Life Science, Shiga University of Medical Science, Otsu City, Shiga, Japan.
Received 24 July 2008; revised 24 August 2008; accepted 24 August 2008; published online 18 September 2008

Human CD46 is expressed on almost all human cells, leading to broad tropism of Ad35 vectors in human cells, including coxsackievirus-adenovirus receptor-negative cells.[8,12] However, intravenous administration of Ad35 vectors resulted in inefficient transduction in the organs of human CD46-transgenic (CD46TG) mice and cynomolgus monkeys, which express CD46 in a pattern similar to that of humans.[13–15] These results indicate that CD46 does not successfully serve as a receptor for intravascularly injected Ad35 vectors and that Ad35 vectors are unsuitable for intravascular transduction. However, this property of Ad35 vectors would suggest a potential advantage, in that unwanted transduction would not occur in organs other than the organs targeted following direct injection of Ad35 vectors when draining from injected sites into the bloodstream. These properties suggest that Ad35 vectors would be suitable for gene transfer by local administration into the organs. In this study, we examined the transduction properties of Ad35 vectors following intraorgan administration in nonhuman primates, that is, cynomologus monkeys.

A previously constructed Ad35 vector expressing β-galactosidase (Ad35LacZ)[15] was locally administered at a dose of 1.5×10^{11} vector particles (VP) per point (high dose) or 3×10^{10} VP per point (low dose) in the following eight organs of two cynomolgus monkeys (designated no. 8 and no. 9; no. 8 received the high dose of Ad35LacZ and no. 9 received the low dose): liver, cerebrum, eyeball (vitreous body), quadriceps femoris muscle, pancreas, kidney, spleen and nasal cavity. Four days after administration, the tissues around the injection sites (approximately $40 \times 40 \times 10$ mm^3 with a central focus at the injection point) were collected and subjected to an analysis of β-galactosidase expression and histological pathology. The health condition of the monkeys was also monitored until necropsy.

Overall, both monkeys did well during the experiment. There were no apparent abnormalities in body temperature or heart rate, although no. 8, the high-dose monkey, exhibited slight reductions in blood pressure and body weight. Both monkeys apparently exhibited increased serum levels of aspartate aminotransferase and creatine phosphokinase on days 0–2 after injection. Mild decreases in hemoglobin levels and increases in levels of lactate dehydrogenase and C-reactive protein were also found in both animals. However, these changes were probably due to the operation. The levels of alanine aminotransferase, alkaline phosphatase, albumin, glucose, calcium, chloride and sodium in the serum were mostly within the normal ranges.

After the direct injection of the Ad35 vectors, the transduction profiles were assessed by immunostaining of β-galactosidase in the tissue sections; Table 1 summarizes the results. A detailed transduction profile in each organ is described below.

Liver

Direct injection of Ad35LacZ to the liver caused tissue damage around the injection site (Figures 1a and b). Infiltration of inflammatory cells, necrotic focus and regenerated bile duct epithelial cells were observed. Immunostaining of the liver sections revealed that hepatocytes were mainly transduced with Ad35LacZ in both no. 8 and no. 9 monkeys (Figures 2a and b). A higher level of β-galactosidase was expressed in the liver of no. 8 than in that of no. 9. The transduced cells were predominantly distributed around the injection point (approximately 1×1 mm^2) and were not found outside the periphery of the injection site. β-galactosidase was not expressed in the liver lobes, which were not injected with Ad35LacZ. β-galactosidase-expressing cells were mainly found on the border region between the normal and damaged areas. Direct injection of naked plasmid DNA or Ad5 vectors into mouse liver also resulted in the localized distribution of transgene-expressing cells around the injection points.[16,17] The liver would not allow dispersion of locally injected Ad vectors in the tissue.

Table 1 β-galactosidase expression in the organs following direct injection of Ad35LacZ into organs

	No. 8 (high dose)	*No. 9 (low dose)*
Liver	+++	+
Cerebrum	+++	+
Eyeball	+	−
Femoral muscle	+	+
Pancreas	−	++
Kidney	−	++
Spleen	−	−
Nasal cavity	−	−

+++, strong positive; ++, moderate positive; +, weakly positive; −, negative.

Cerebrum

Ad35LacZ was stereotaxically injected into the left frontal lobe of the cerebrum. After infusion of the high dose of Ad35LacZ, softening of the tissue, which appeared necrotic, was widely observed in the left basal ganglia (Figure 1c). Neutrophils were infiltrated into the necrotic area. In contrast, injection of a low dose of Ad35LacZ resulted in no apparent toxicity, although slight bleeding was found around the artery (Figure 1d). Transduced cells, which appeared to be microglia, were found around the softening regions of both no. 8 and no. 9 animals, although the latter had fewer transduced microglia (Figures 2c and d). There were no β-galactosidase-expressing cells in the right hemisphere of the brain, which was infused with phosphate-buffered saline buffer (data not shown).

Eye

Ad35LacZ was infused into the vitreous body for inoculation into the eyeball. The high dose induced invasion by inflammatory cells, including macrophages and neutrophils, into the ciliary body, iris and retina (Figure 1e). Necrotic changes were also found in all layers of the retina. The low dose caused similar damage to the eyeball. The high dose mediated transduction in the conjunctival epithelial cells (Figure 2e). β-galactosidase expression was not observed in other areas. After injection into the vitreous body, Ad35LacZ might be drained from it and transduce the conjunctival epithelial cells. Bora *et al.*[18] demonstrated that human CD46 was hardly expressed in eye tissues, suggesting that these tissues are refractory to Ad35 vectors. We did not find β-galactosidase expression in the eye of no. 9 animal. Phosphate-buffered saline injection did not result in

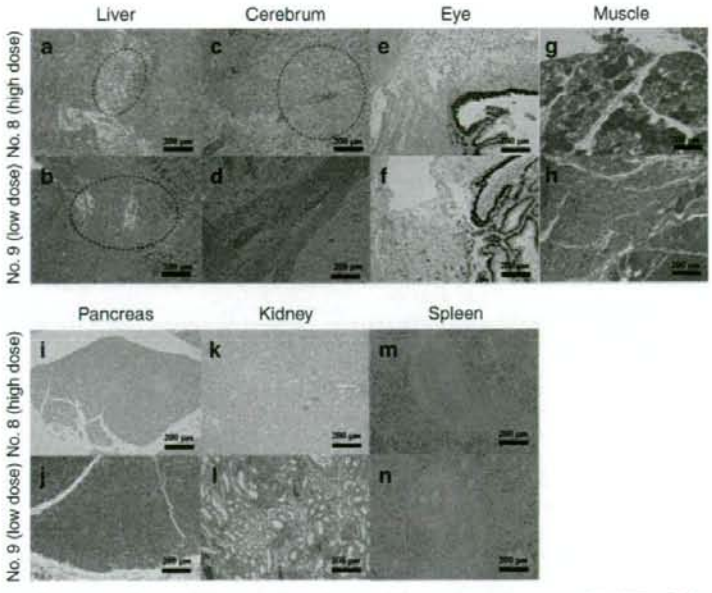


Figure 1 Tissue histology in the organs of cynomolgus monkeys 4 days after intraorgan injection of Ad35LacZ. (**a** and **b**) The liver, (**c** and **d**) cerebrum, (**e** and **f**) eyeball, (**g** and **h**) skeletal muscle, (**i** and **j**) pancreas, (**k** and **l**) kidney and (**m** and **n**) spleen. Young male cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and with the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The animals (approximately 3 years of age, 1.9 and 2.2 kg) were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpesvirus B, varicella-zoster-like virus and measles virus. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Osaka, Japan). The liver, cerebrum, eyeball, nasal cavity, pancreas, kidney, skeletal muscle and spleen of cynomolgus monkeys were each injected with Ad35LacZ suspended in 200 µl (100 µl for eyeball) of phosphate-buffered saline at a dose of 1.5×10^{11} vector particles (VP) per point (monkey no. 8) or 3×10^{10} VP per point (monkey no. 9). Four days after injection, tissue sections were hematoxylin–eosin stained by a routine method. Dotted-line circles in (**b**) and (c) indicate the necrotic area in the liver and the softening area in the cerebrum, respectively.

transgene expression or apparent abnormality in the eyeball (data not shown).

Femoral muscle

Severe inflammation did not occur after intramuscular injection of the high dose, although we found slight damage to the muscle fibers (Figure 1g). In contrast, the low dose induced more severe inflammation (Figure 1h). Infiltration of neutrophils and macrophages was seen in the muscle of no. 9. It is currently unclear why the low dose induced higher levels of damage. A slight difference in the injection point might affect Ad35 vector-induced inflammatory responses in the muscle. β-galactosidase expression was found only in the cells that appeared to be macrophages and/or fibroblasts located among the muscle fibers in both monkeys (Figures 2g and h). No muscle fibers expressed β-galactosidase in either monkey. It remains to be elucidated why intramuscular injection of Ad35 vectors mediated poor transduction in muscle fibers of cynomolgus monkeys. Ad35 vectors transduced the muscle following intramuscular injection in wild-type mice and in CD46TG mice.[12,14] The transduction mechanism and efficiencies of Ad35 vectors in muscle fibers might differ among species, and the muscle of nonhuman primates might be more refractory to transduction than that of rodents. Thirion *et al*.[19] demonstrated that Ad vectors would transduce human, rat and mouse primary muscle cells through different pathways. Danko *et al*.[20] reported that transgene expression levels by intramuscular injection of naked DNA were lower in dogs and nonhuman primates than in rodents. On the other hand, several studies demonstrated the utility of Ad35 vectors as vaccine vectors that express antigen by intramuscular administration in mice and nonhuman primates.[3,4] Macrophages and/or dendritic cells transduced with Ad35 vectors might play important roles in transgene-specific immune responses by intramuscular injection of Ad35 vectors.

Pancreas

Injection into the pancreas caused no severe damage to that organ in either monkey (Figures 1i and j). We did not find transduced cells in the pancreas of no. 8; in contrast, β-galactosidase was apparently expressed in exocrine acinar cells of no. 9 in the pancreatic lobules (Figures 2i and j). Chemiluminescence assay of β-galactosidase also revealed significant levels of β-galactosidase expression in the pancreas of no. 9 but not in that of no. 8 (data not shown). Wang *et al*.[21] also demonstrated that direct injection of conventional Ad vectors and adenoassociated virus vectors into murine pancreas achieved efficient transduction in acinar cells. Pancreatic acinar cells would be susceptible to Ad vectors.

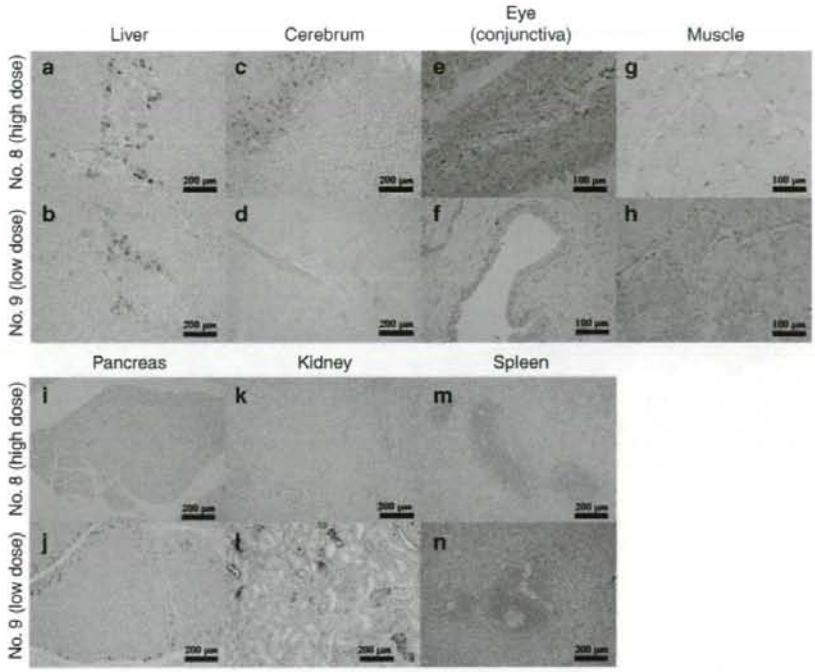


Figure 2 β-galactosidase expression in the organs of cynomolgus monkeys 4 days after intraorgan injection of Ad35LacZ. (**a** and **b**) The liver, (**c** and **d**) cerebrum, (**e** and **f**) eyeball, (**g** and **h**) skeletal muscle, (**i** and **j**) pancreas, (**k** and **l**) kidney and (**m** and **n**) spleen. Ad35LacZ was locally administered in the organs of cynomolgus monkeys at the low (3×10^{10} vector particles (VP) per point) or high dose (1.5×10^{11} VP per points) as described in Figure 1. Four days after injection, the tissues were collected for analysis of β-galactosidase expression and histological pathology. Immunostaining of β-galactosidase was performed using anti-β-galactosidase antibody (Abcam, Cambridge, UK).

Kidney

Ad35LacZ injection to the left kidney induced infiltration by inflammatory cells, including lymphocytes, into the interstitial tissue of the kidney (Figures 1k and l). The right kidney, which was injected with phosphate-buffered saline, did not exhibit β-galactosidase expression or inflammatory responses (data not shown). The high dose did not mediate β-galactosidase expression, but the low dose led to apparent transduction (Figures 2k and l). The renal tubular epithelial cells were mainly transduced with Ad35LacZ. In the kidney, compared with the other organs, transduced cells were more widely spread around the injection points. Refractoriness to the high dose and massive β-galactosidase expression by the low dose in the pancreas and kidney together form a major conundrum in this study. The differences in transduction efficiencies might be due to the slight differences in injection sites. Especially, Ad35LacZ may have been drained into the renal tubule of no. 9 following injection into the kidney, leading to efficient transduction in the renal tubule epithelial cells. Ad35 was originally identified in the kidney and causes cystitis,[22] indicating the tropism of Ad35 for renal epithelial cells.

Spleen and nasal cavity

Unexpectedly, direct injection of Ad35LacZ to the spleen did not induce inflammatory responses such as hyperplasia (Figures 1m and n). There was no β-galactosidase expression in the spleen of either monkey (Figures 2m and n). For transduction in the mucosal membrane of the nasal cavity, Ad35 vector suspensions were instilled into the nasal cavity of each monkey, but neither one showed β-galactosidase expression or cellular damage in the mucosal membrane of the nasal cavity (data not shown).

Other organs

β-galactosidase production in the lung, heart, thymus, bone marrow, lymph node, bladder and testis, which were not injected with Ad35LacZ, were examined by chemiluminescence assay. None of these organs showed detectable β-galactosidase expression (data not shown).

Next, we determined the blood concentrations of Ad35LacZ genome DNA in the blood using quantitative real-time PCR to examine whether or not Ad35LacZ locally injected to the organs was drained from the injection site into the bloodstream. The Ad35 vector DNA was detected in the blood as soon as 6 h post-injection, then gradually decreased (Figure 3). However, the blood-clearance kinetics of Ad35LacZ following intraorgan injection were slower than those following intravenous administration, which were previously reported,[23] although the total amounts of Ad35 vector doses in this study (no. 8: 1.5×10^{11} VP × 8 points; no. 9: 3×10^{10} VP × 8 points) were comparable to or lower than those in the previous study in which Ad35LacZ was intravenously infused in cynomolgus monkeys ($0.4–2 \times 10^{12}$ VP per kg, 1.88–2.96 kg).[23] Ad35 vector

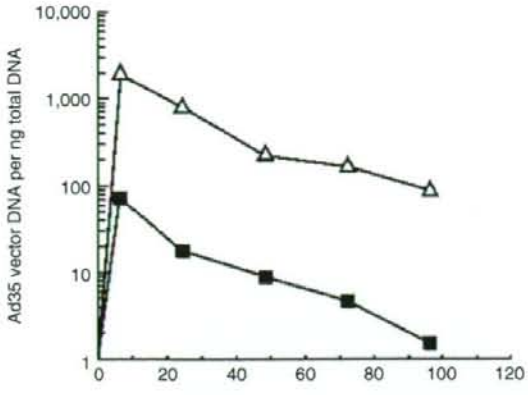


Figure 3 Blood concentrations of Ad35 vectors in cynomolgus monkeys following intraorgan administration. Ad35LacZ was locally administered in the organs of cynomolgus monkeys at the low (3×10^{10} vector particles (VP) per point, closed square) or high dose (1.5×10^{11} VP per points, open triangle) as described in Figure 1. Blood was collected at the indicated post-injection time points (6, 24, 48, 72 and 96 h post-injection). Total DNA, including Ad vector DNA, was isolated from the blood, and the Ad vector DNA contents were measured by quantitative TaqMan PCR assay, as previously described.[23]

DNA was still detectable 4 days after injection. These results suggest that Ad35 vectors or Ad35 vector DNA remaining in the injection sites might be gradually released from the injection sites and drained into the bloodstream.

Furthermore, to examine whether or not Ad35LacZ draining into the bloodstream was accumulated in the organs, we determined the Ad35 DNA contents in the portions of the liver and spleen that were away from the respective injection sites. The liver and spleen play crucial roles in the clearance of systemically injected Ad vectors. The Ad35 vector DNA was not detected in those portions of the liver in no. 9, but was detected in the portions of the liver in no. 8 and in those of the spleen in both monkeys (data not shown). These results suggest that Ad35LacZ or the Ad35 vector DNA draining into the systemic circulation would be taken up by the liver and spleen. We further assessed the Ad35 DNA contents in the lungs, heart, thymus and bone marrow, in which Ad35 vectors were not directly infused. Ad35 vector DNA was detected in the lungs and heart of no. 8 but not in those of no. 9 (data not shown). We did not detect Ad35 vector DNA in the thymus or bone marrow of either monkey. Considering that intravascularly injected Ad35 vectors did not efficiently transduce organs,[15] organs must not be transduced with Ad35LacZ, which is drained into the bloodstream and taken up by the organs.

In most cases of cancer gene therapy using Ad vectors, the vectors are administered directly to the tumor regions.[24–26] When used as vaccine vectors, on the other hand, Ad vectors are intramuscularly injected.[27,28] In addition, Ad vectors are intramyocardially injected in angiogenic gene therapy.[29,30] Thus, direct infusion of Ad vectors to organs is one of the most frequent application methods in clinical settings. However, there has been little information about the transduction properties of these vectors following direct injection into organs. This study demonstrated that different types of cells were transduced with Ad35 vectors depending on the organ after direct infusion into the organ. The differences in the histological structures and cell types comprising the organs would explain the differences in transduction properties of locally injected Ad35 vectors. This study provides important information for clinical study by intraorgan injection of Ad35 vectors, although the characteristics of the organs (structure, cell types and so on) differ different between normal tissue and diseased areas.

Acknowledgements

We thank Fumiko Ono and Chieko Ohno (The Corporation for Production and Research of Laboratory Primates, Ibaraki, Japan) for their help. This study was supported by grants from the Ministry of Health, Labour, and Welfare of Japan and by a Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

References

1 Havenga MJ, Lemckert AA, Ophorst OJ, van Meijer M, Germeraad WT, Grimbergen J *et al.* Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. *J Virol* 2002; **76**: 4612–4620.

2 De Jong JC, Wermenbol AG, Verweij-Uijterwaal MW, Slaterus KW, Wertheim-Van Dillen P, Van Doornum GJ *et al.* Adenoviruses from human immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51 of species B1 and D, respectively. *J Clin Microbiol* 1999; **37**: 3940–3945.

3 Lemckert AA, Sumida SM, Holterman L, Vogels R, Truitt DM, Lynch DM *et al.* Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol* 2005; **79**: 9694–9701.

4 Nanda A, Lynch DM, Goudsmit J, Lemckert AA, Ewald BA, Sumida SM *et al.* Immunogenicity of recombinant fiber-chimeric adenovirus serotype 35 vector-based vaccines in mice and rhesus monkeys. *J Virol* 2005; **79**: 14161–14168.

5 Mizuguchi H, Hayakawa T. Enhanced antitumor effect and reduced vector dissemination with fiber-modified adenovirus vectors expressing herpes simplex virus thymidine kinase. *Cancer Gene Ther* 2002; **9**: 236–242.

6 Okada Y, Okada N, Mizuguchi H, Hayakawa T, Mayumi T, Mizuno N. An investigation of adverse effects caused by the injection of high-dose TNFalpha-expressing adenovirus vector into established murine melanoma. *Gene Therapy* 2003; **10**: 700–705.

7 Suzuki T, Sakurai F, Nakamura S, Kouyama E, Kawabata K, Kondoh M *et al.* miR-122a-regulated expression of a suicide gene prevents hepatotoxicity without disturbing the antitumor effects in suicide gene therapy. *Mol Ther* 2008, (in press).

8 Sakurai F, Mizuguchi H, Hayakawa T. Efficient gene transfer into human CD34+ cells by an adenovirus type 35 vector. *Gene Therapy* 2003; **10**: 1041–1048.

9 Vogels R, Zuijdgeest D, van Rijnsoever R, Hartkoorn E, Damen I, de Bethune MP *et al.* Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol* 2003; **77**: 8263–8271.

10 Gao W, Robbins PD, Gambotto A. Human adenovirus type 35: nucleotide sequence and vector development. *Gene Therapy* 2003; **10**: 1941–1949.
11 Seshidhar Reddy P, Ganesh S, Limbach MP, Brann T, Pinkstaff A, Kaloss M *et al*. Development of adenovirus serotype 35 as a gene transfer vector. *Virology* 2003; **311**: 384–393.
12 Sakurai F, Mizuguchi H, Yamaguchi T, Hayakawa T. Characterization of *in vitro* and *in vivo* gene transfer properties of adenovirus serotype 35 vector. *Mol Ther* 2003; **8**: 813–821.
13 Sakurai F, Kawabata K, Koizumi N, Inoue N, Okabe M, Yamaguchi T *et al*. Adenovirus serotype 35 vector-mediated transduction into human CD46-transgenic mice. *Gene Therapy* 2006; **13**: 1118–1126.
14 Verhaagh S, de Jong E, Goudsmit J, Lecollinet S, Gillissen G, de Vries M *et al*. Human CD46-transgenic mice in studies involving replication-incompetent adenoviral type 35 vectors. *J Gen Virol* 2006; **87**: 255–265.
15 Sakurai F, Nakamura S, Akitomo K, Shibata H, Terao K, Kawabata K *et al*. Transduction properties of adenovirus serotype 35 vectors after intravenous administration into nonhuman primates. *Mol Ther* 2008; **16**: 726–733.
16 Sakai M, Nishikawa M, Thanaketpaisarn O, Yamashita F, Hashida M. Hepatocyte-targeted gene transfer by combination of vascularly delivered plasmid DNA and *in vivo* electroporation. *Gene Therapy* 2005; **12**: 607–616.
17 Crettaz J, Berraondo P, Mauleon I, Ochoa L, Shankar V, Barajas M *et al*. Intrahepatic injection of adenovirus reduces inflammation and increases gene transfer and therapeutic effect in mice. *Hepatology* 2006; **44**: 623–632.
18 Bora NS, Gobleman CL, Atkinson JP, Pepose JS, Kaplan HJ. Differential expression of the complement regulatory proteins in the human eye. *Invest Ophthalmol Vis Sci* 1993; **34**: 3579–3584.
19 Thirion C, Lochmuller H, Ruzsics Z, Boelhauve M, Konig C, Thedieck C *et al*. Adenovirus vectors based on human adenovirus type 19a have high potential for human muscle-directed gene therapy. *Hum Gene Ther* 2006; **17**: 193–205.
20 Danko I, Williams P, Herweijer H, Zhang G, Latendresse JS, Bock I *et al*. High expression of naked plasmid DNA in muscles of young rodents. *Hum Mol Genet* 1997; **6**: 1435–1443.
21 Wang AY, Peng PD, Ehrhardt A, Storm TA, Kay MA. Comparison of adenoviral and adeno-associated viral vectors for pancreatic gene delivery *in vivo*. *Hum Gene Ther* 2004; **15**: 405–413.
22 Hierholzer JC. Adenoviruses in the immunocompromised host. *Clin Microbiol Rev* 1992; **5**: 262–274.
23 Sakurai F, Nakamura S, Akitomo K, Shibata H, Terao K, Hayakawa T *et al*. Transduction properties of adenovirus serotype 35 vectors after intravenous administration in non-human primates. *Mol Ther* 2008; **16**: 726–733.
24 Shirakawa T, Terao S, Hinata N, Tanaka K, Takenaka A, Hara I *et al*. Long-term outcome of phase I/II clinical trial of Ad-OC-TK/VAL gene therapy for hormone-refractory metastatic prostate cancer. *Hum Gene Ther* 2007; **18**: 1225–1232.
25 Shimada H, Matsubara H, Shiratori T, Shimizu T, Miyazaki S, Okazumi S *et al*. Phase I/II adenoviral p53 gene therapy for chemoradiation resistant advanced esophageal squamous cell carcinoma. *Cancer Sci* 2006; **97**: 554–561.
26 Tong AW, Nemunaitis J, Su D, Zhang Y, Cunningham C, Senzer N *et al*. Intratumoral injection of INGN 241, a nonreplicating adenovector expressing the melanoma-differentiation associated gene-7 (mda-7/IL24): biologic outcome in advanced cancer patients. *Mol Ther* 2005; **11**: 160–172.
27 Catanzaro AT, Koup RA, Roederer M, Bailer RT, Enama ME, Moodie Z *et al*. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. *J Infect Dis* 2006; **194**: 1638–1649.
28 Rosenberg SA, Zhai Y, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM *et al*. Immunizing patients with metastatic melanoma using recombinant adenoviruses encoding MART-1 or gp100 melanoma antigens. *J Natl Cancer Inst* 1998; **90**: 1894–1900.
29 Stewart DJ, Hilton JD, Arnold JM, Gregoire J, Rivard A, Archer SL *et al*. Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. *Gene Therapy* 2006; **13**: 1503–1511.
30 Rosengart TK, Lee LY, Patel SR, Kligfield PD, Okin PM, Hackett NR *et al*. Six-month assessment of a phase I trial of angiogenic gene therapy for the treatment of coronary artery disease using direct intramyocardial administration of an adenovirus vector expressing the VEGF121 cDNA. *Ann Surg* 1999; **230**: 466–470; discussion 470–472.

Transduction Properties of Adenovirus Serotype 35 Vectors After Intravenous Administration Into Nonhuman Primates

Fuminori Sakurai[1], Shin-ichiro Nakamura[2,3], Kimiyo Akitomo[1], Hiroaki Shibata[2], Keiji Terao[2], Kenji Kawabata[1], Takao Hayakawa[4] and Hiroyuki Mizuguchi[1,5]

[1]Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Ibaraki City, Osaka, Japan; [2]Tsukuba Primates Research Center, National Institute of Biomedical Innovation, Tsukuba City, Ibaraki, Japan; [3]The Corporation for Production and Research of Laboratory Primates, Tsukuba City, Ibaraki, Japan; [4]Pharmaceuticals and Medical Devices Agency, Chiyoda-Ku, Tokyo, Japan; [5]Graduate School of Pharmaceutical Sciences, Osaka University, Suita City, Osaka, Japan

Adenovirus serotype 35 (Ad35) vectors have shown promise as effective gene delivery vehicles. However, the transduction profiles of Ad35 vectors in conventional mice allow only a limited estimation of transduction properties of these vectors, because the mouse analog of the subgroup B Ad receptor, CD46, is restricted to the testis. In order to assess the transduction properties of Ad35 vectors more completely, we performed transduction experiments using cynomolgus monkeys, which ubiquitously express CD46 in a pattern similar to that in humans. *In vitro* transduction experiments demonstrated that cultured cells from the cynomolgus monkey were efficiently transduced with Ad35 vectors. In contrast, after intravenous administration into live monkeys hardly any evidence of Ad35 vector–mediated transduction was found in any of the organs, although Ad35 vector genomes were detected in various organs. Less severe histopathological abnormalities were found in the Ad35 vector–infused monkeys than in the conventional Ad5 vector–injected monkeys. In the latter, serious tissue damage and inflammatory responses, such as hepatocyte necrosis and lymphatic hyperplasia in the colon, were induced. Both Ad35 and Ad5 vectors caused similar hematological changes (increase in $CD3^+$ cells, and decrease in $CD16^+$ cells and $CD20^+$ cells) in peripheral blood cells. These results should provide valuable information for the clinical application of Ad35 vectors.

Received 15 September 2007; accepted 16 January 2007; advance online publication 11 March 2008. doi:10.1038/mt.2008.19

INTRODUCTION

Human adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses that are composed of 51 serotypes.[1,2] Among the 51 serotypes, the conventional Ad vectors that are most widely used, including for human clinical trials, are constructed based on the subgroup C Ad serotype 5 (Ad5). Ad5 vectors have several advantages as gene delivery vehicles, but clinical and preclinical studies have revealed three major disadvantages of Ad5 vectors. First, target cells that are important for gene therapy, including malignant tumor cells and dendritic cells, express nil or insufficient levels of a cellular receptor for Ad5, the coxsackievirus-adenovirus receptor. The transduction efficiencies of Ad5 vectors depend to a large extent on the expression levels of coxsackievirus-adenovirus receptor, leading to refractoriness of coxsackievirus-adenovirus receptor-negative cells to Ad5 vectors.[3] Second, >50% of adults are seropositive for Ad5 because natural infection with Ad5 is common.[4,5] Pre-existing anti-Ad5 antibodies not only largely inhibit Ad5 vector–mediated transduction, but may also enhance the toxicities induced by Ad5 vectors.[6] Third, inflammatory responses are systemically and rapidly induced by intravascular administration of Ad5 vectors, leading to tissue damage, and this can be fatal to the host.[7–10]

In order to address these problems, we as well as others have developed a replication-incompetent subgroup B Ad serotype 35 (Ad35) vector.[11–15] Ad35 vectors utilize human CD46 as a cellular receptor.[16,17] Human CD46 is ubiquitously expressed on almost all human cells, leading to a wide tropism of Ad35 vectors. In addition, pre-existing anti-Ad5 immunity does not hamper Ad35 vector–mediated transduction, and seroprevalence for Ad35 is much lower than that for Ad5 (refs. 13,14). Ad35 vectors have properties that make them very promising prospects for use as transduction vehicles, but the transduction efficiencies of Ad35 vectors in conventional mice are lower than those of Ad5 vectors.[12,14] Conventional mice seem inappropriate as animal models for Ad35 vectors because mouse CD46 is expressed only in the testis.[18] In addition, there is low homology between human CD46 and mouse CD46. We considered that transduction experiments with Ad35 vectors should be performed using nonhuman primates so as to properly evaluate the transduction properties of Ad35 vectors. The CD46 of nonhuman primates is ubiquitously expressed in a similar pattern to humans, and shows high homology to human CD46.[19]

In this study, we examined the transduction profiles of Ad35 vectors after intravenous administration into nonhuman primates, *i.e.*, cynomolgus monkeys. Ad35 vector–induced immune responses and the blood concentrations of Ad35 vectors were

Correspondence: *Hiroyuki Mizuguchi, Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Asagi, Saito, Ibaraki City, Osaka 567-0085, Japan. E-mail: mizuguch@nibio.go.jp*

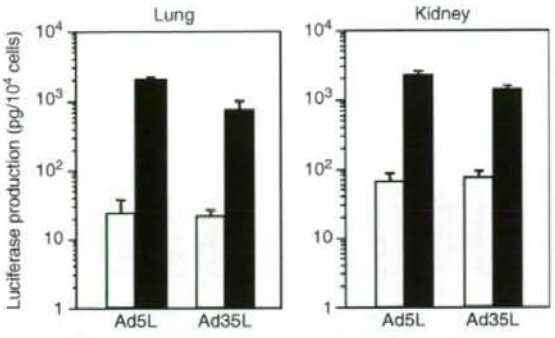


Figure 1 *In vitro* transduction efficiencies of Ad35 and Ad5 vectors in cultured cells of cynomolgus monkey. Luciferase production in primary lung and kidney cells following Ad vector transduction. Primary lung and kidney cells isolated from cynomolgus macaque embryos were transduced with Ad35L or Ad5L at 300 (open bar) and 3,000 vector particles/cell (closed bar) for 1.5 hours. After a 48-hour culture, luciferase production in the cells was measured by luminescence assay. The data are expressed as the mean values ± SD ($n = 4$). Luciferase expression in the mock-infected cells was less than the detectable level. Ad, adenovirus.

analyzed for 4 days after the injection. Necropsy was performed 4 days after the injection to examine the transduction efficiencies, tissue accumulations of Ad35 vectors, and histopathological changes in the organs after injection.

RESULTS

In vitro transduction in cultured cynomolgus monkey cells

First, to examine whether cynomolgus monkey cells were susceptible to Ad35 vectors, primary lung and kidney cells isolated from embryonic cynomolgus monkeys were transduced with a firefly luciferase–expressing Ad35 vector (Ad35L) and a conventional Ad5 vector (Ad5L). Both Ad35L and Ad5L vectors were shown to mediate efficient transduction in the cells from both organs (**Figure 1**). Ad35 vectors also efficiently transduced the cynomolgus monkey T-cell line HSC-F (**Supplementary Figure S1**). These results indicate that cynomolgus monkey cells are susceptible to Ad35 vectors. However, peripheral blood mononuclear cells of cynomolgus monkeys were almost refractory to Ad35 vectors (data not shown).

Blood clearance of Ad vectors

Next, the six cynomolgus monkeys (designated #1–#6) were administered either a β-galactosidase-expressing Ad35 vector (Ad35LacZ) or an Ad5 vector (Ad5LacZ) through the femoral vein (**Supplementary Table S1**). The blood clearances of the Ad vectors were examined using a quantitative real-time polymerase chain reaction. Both Ad35LacZ and Ad5LacZ vectors were rapidly cleared from the blood circulation within 24 hours after the injection (**Figure 2a** and **b**). We did not find any apparent differences between the blood-clearance kinetics of Ad35LacZ and Ad5LacZ. Assuming that the entire Ad vector DNA in the blood was completely recovered from the blood samples, there would remain 0.12% and 0.09% of the injected Ad35LacZ in the blood of monkey #6 at 3 and 6 hours after injection, respectively. The lower levels of

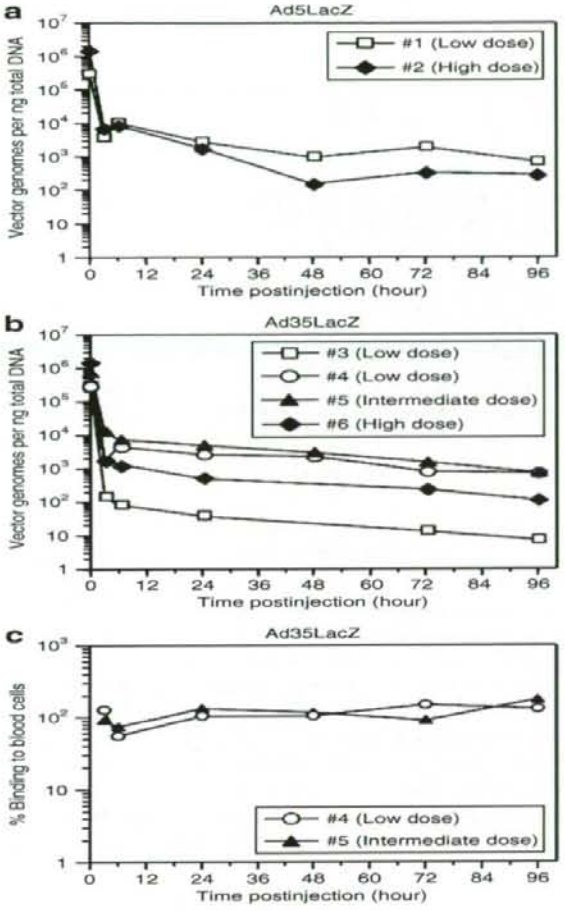


Figure 2 Persistence of adenoviral (Ad) vectors in the blood of cynomolgus monkeys following systemic administration. (**a**) Ad vector DNA concentrations in the blood after intravenous administration. Cynomolgus monkeys were intravenously infused with Ad35LacZ or Ad5LacZ at low [0.4×10^{12} vector particles (VP)/kg], intermediate (1.0×10^{12}VP/kg), or high (2×10^{12}VP/kg) doses. Blood was collected at the indicated time points after injection (3, 6, 24, 48, 72, and 96 hours after injection). Total DNA, including Ad vector DNA, was isolated from the blood, and the Ad vector DNA contents were measured using quantitative TaqMan polymerase chain reaction (PCR) assay. The concentrations of the Ad vectors in the blood at the zero time point were calculated based on the total number of Ad vector particles infused and the estimated circulating blood volume (65 ml/kg). Ad vector DNA was not detected in the blood before injection. (**b**) Percentages of blood cell–associated Ad35lacZ remaining in the blood after systemic administration in cynomolgus monkeys. After isolating the blood as described, blood cells were washed twice with phosphate-buffered saline buffer and the amounts of Ad35LacZ associated with blood cells were evaluated using TaqMan PCR as described earlier. The percentages were calculated as follows: 100 × (the amounts of Ad35 vector DNA associated with blood cells)/(the amounts of Ad35 vector DNA recovered from whole blood).

Ad35LacZ remaining in the blood of monkeys #3 and #6 than those in monkeys #4 and #5 might have been partly because of the low infectious titer-to-particle ratio of the vector batch of Ad35LacZ injected into monkeys #3 and #6. The infectious titer-to-particle

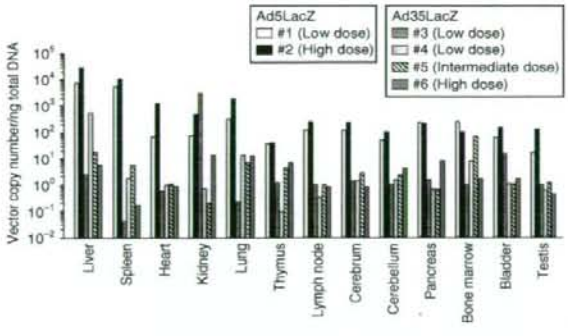


Figure 3 *In vivo* tissue distributions of adenoviral (Ad) vector DNA in cynomolgus monkeys after systemic administration. Ad35LacZ or Ad5LacZ was intravenously administered into cynomolgus monkeys as described for **Figure 2**. Four days after the injection, necropsy was performed, and Ad vector DNA contents were measured using quantitative TaqMan polymerase chain reaction analysis. The Ad vector DNA was not detected in the organs of mock-infected animals.

ratio of the Ad35LacZ used in monkeys #3 and #6 was lower than that used in monkeys #4 and #5 (data not shown). Noninfectious Ad particles might be more easily degraded in the blood or taken up by phagocytic cells.

Further, we examined whether the Ad35 vectors were associated with blood cells in the blood stream after the injection. The majority of Ad35LacZ remaining in the blood was associated with blood cells at all the time points (**Figure 2c**). Similarly, assuming the complete recovery of the Ad vector DNA as described earlier, 1.5% of the injected Ad35LacZ would be associated with blood cells in monkey #5 at 3 hours after the injection. The levels of Ad35LacZ associated with blood cells remained constant during the study. These results suggest that Ad35 vectors may bind to blood cells, or be taken up by blood cells after the injection.

Tissue distribution of Ad vectors

In order to examine the biodistribution of Ad35 and Ad5 vectors in cynomolgus monkeys after intravenous administration, Ad DNA contents in the organs were assessed (**Figure 3**). The Ad35 vector DNA was mainly found in the liver, lung, and kidney; however, the levels of Ad35 vector DNA were one to five orders of magnitude lower in almost all organs than the levels of Ad5 vector DNA, which was found mainly in the liver and spleen. Ad35LacZ was also less efficiently accumulated in the organs that exhibited low levels of Ad5LacZ accumulation, such as the thymus and testis.

Ad vector–mediated transgene expression in organs

In order to evaluate the *in vivo* transduction efficiencies of Ad35 and Ad5 vectors, β-galactosidase expression in the organs was examined. Ad5LacZ efficiently transduced the organs (**Figure 4a**). The highest level of β-galactosidase production was found in the liver, followed by the spleen. Liver parenchymal cells and spleen marginal zone cells were mainly transduced by Ad5LacZ in these organs (**Figure 4b**). On the other hand, Ad35 vector–mediated β-galactosidase expression in the organs at all doses was approximately equal to, or slightly above, the levels in

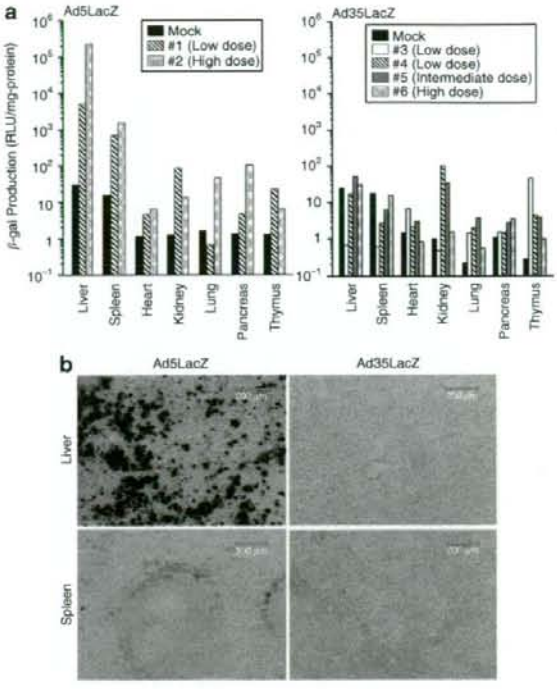


Figure 4 Adenoviral (Ad) vector–mediated transgene expression in cynomolgus monkeys after systemic administration. (**a**) Chemiluminescence analysis of β-galactosidase production in cynomolgus monkeys after systemic administration of Ad35LacZ or Ad5LacZ. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys as described for **Figure 2**. Four days after injection, the organs were collected, and β-galactosidase production in the organs was assessed using a chemiluminescence assay. (**b**) X-gal staining of tissue sections of cynomolgus monkeys receiving Ad5LacZ or Ad35LacZ. Four days after intravenous administration of Ad35LacZ or Ad5LacZ at a high dose (2×10^{12} vector particles/kg), tissues were collected, and X-gal staining was performed as described in Materials and Methods. RLU, relative light units.

mock-infected animals. X-gal-positive cells were not found in the tissue sections of the liver or spleen of the Ad35LacZ-infused monkeys. These results indicate that Ad35 vectors show much lower transduction activity than Ad5 vectors after systemic delivery in cynomolgus monkeys.

Serum chemistry profiles

Next, we measured the levels of serum biochemical markers to assess Ad vector–induced tissue/organ damage. Almost all the markers were increased following Ad vector injection; however, overall, the markers examined appeared to be more elevated in the monkeys receiving Ad5LacZ than in those receiving Ad35LacZ (**Figure 5a**). Aspartate aminotransferase (AST) levels were elevated as early as 3 hours after the injection, and peaked at 24 hours in most cases. The peak levels of AST in Ad35LacZ-injected monkeys #3, #4, #5, and #6 were 6.1-, 4.8-, 8.2-, and 3.8-fold higher than the preinjection levels, respectively. By contrast, Ad5LacZ-infused monkeys (#1 and #2) showed 4.9- and 27.5-fold increases in AST at the peak points, respectively. Significant elevations in alanine

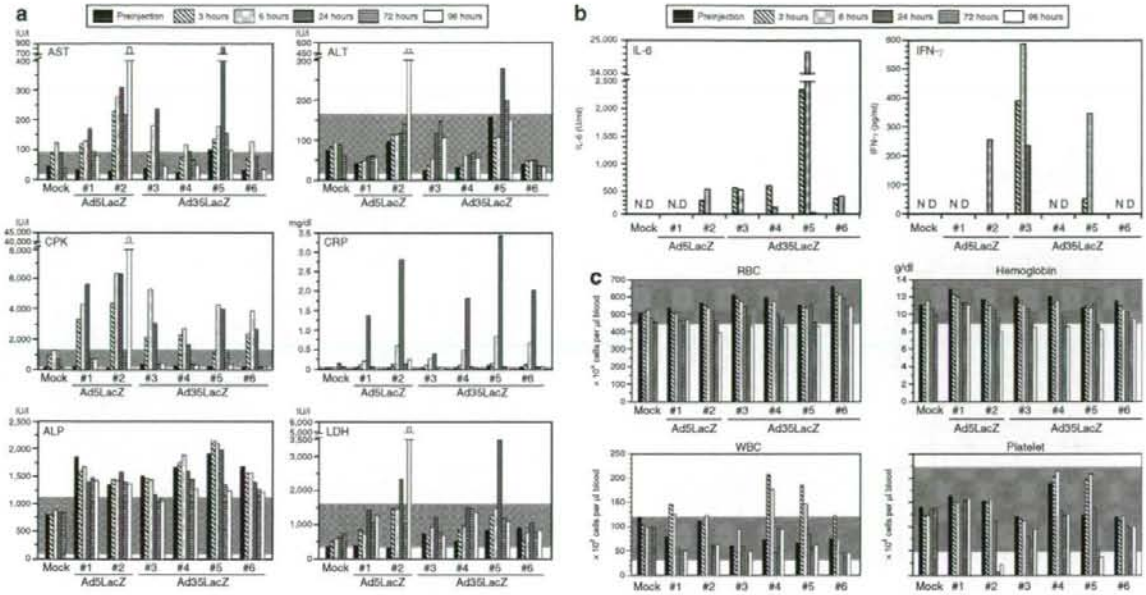


Figure 5 Blood analysis after adenoviral (Ad) vector administration to cynomolgus monkeys. (**a**) Serum marker levels, (**b**) inflammatory cytokine productions, and (**c**) blood cell counts in the peripheral blood after Ad vector administration. The gray area in the graphs of serum markers and blood cell counts indicates the normal range for adult cynomolgus monkeys. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys and blood was collected as described for **Figure 2**. Serum marker levels and blood cell counts were measured using routine methods. Inflammatory cytokine levels were examined using enzyme-linked immunosorbent assay. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatine phosphokinase; CRP, C-reactive protein; IFN-γ, interferon-γ; IL-6, interleukin-6; LDH, lactate dehydrogenase; ND, not detected (under the limit of detection); RBC, red blood cell; WBC, white blood cell.

aminotransferase were also found in several of the monkeys, but the alanine aminotransferase levels were within the normal range at almost all the time points. Creatine phosphokinase (CPK) levels sharply rose to a peak 6 or 24 hours after injection. CPK in the Ad35 vector–injected monkeys #3, #4, #5, and #6 showed 14.2-, 9.7-, 16.3-, and 17.7-fold increases at the peak points. On the other hand, the Ad5 vector–injected monkeys #1 and #2 exhibited 16.6- and 40.9-fold elevations in CPK at 6 hours after the injection. Dramatic increases in AST, alanine aminotransferase, and CPK levels in monkey #2 at 96 hours after injection was possibly caused by a slight expression of Ad5 E2 and/or E4 proteins. E4 protein was expressed in the liver 4 days after injection of conventional Ad vectors in mice, leading to liver damage.[20] Levels of C-reactive protein were also sharply increased in all the Ad vector–injected animals. A high dose of Ad35LacZ and Ad5LacZ caused 29-fold (#6) and 56.2-fold (#2) increases in C-reactive protein levels 24 hours after injection, respectively. Alkaline phosphatase levels gradually decreased over the first 96 hours after injection. Alkaline phosphatase levels at preinjection were higher than the normal range in the monkeys. This is because young cynomolgus monkeys (<4 years of age) often have alkaline phosphatase levels >1,000 IU/L. Apparent increases in lactate dehydrogenase were found in monkeys #2 and #5. The lactate dehydrogenase levels in the other animals were within the normal range. There were no abnormalities in the other parameters, including serum albumin, glucose, cholesterol, calcium, sodium, potassium, and chloride (data not shown).

Inflammatory cytokine induction

In order to examine the innate immune responses after Ad vector injection, inflammatory cytokine levels in the serum were measured (**Figure 5b**). Interleukin-6 (IL-6) was rapidly induced with a peak at 3 or 6 hours after the injection in all the animals except in monkey #1. There were no apparent differences in IL-6 levels between Ad35LacZ-treated and Ad5LacZ-treated animals, except that monkey #5 produced an extremely high level of IL-6. The levels of interferon-γ were also elevated and reached a peak at 6 hours after the injection in monkeys #2, #3, and #5. Tumor necrosis factor-α was not detected in any of the animals (data not shown).

Hematological profiles

In order to evaluate the influence of Ad vector injection on the hematological profiles, we examined the changes in peripheral blood cell counts (**Figure 5c**). The changes in the levels of red blood cells and hemoglobin were marginal, but the levels gradually decreased after injection in all the monkeys, including a mock-infected animal, probably because of the collection of large volumes of blood samples (>5 ml/time point) every day. Ad35LacZ-injected monkeys #3, #4, and #5, and Ad5LacZ-injected monkey #2 showed a rapid decline in platelet levels beginning at 24 hours after the injection. A transient increase in the platelet levels was found 3 and 6 hours after the injection in monkey #5. It remains unclear why the platelet levels increased in monkey #5; however, the previous study also reported an initial increase in the platelet levels after Ad5 vector injection in nonhuman primates.[21] A rapid