A VESE APOPTÓTIKUS ENDONUKLEÁZAINAK SZEREPE A BEJUTTATOTT IDEGEN DNS/RNS LEBONTÁSÁBAN

Egyetemi doktori (PhD) értekezés

Szerző: Buzder Tímea

Témavezető: Dr. Bánfalvi Gáspár

Debreceni Egyetem
Természettudományi Doktori Tanács
Juhász Nagy Pál Doktori Iskola
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Debrecen, 2010 . . . . . .

a jelölt aláírása

Tanúsítom, hogy Buzder Tímea doktorjelölt 2007- 2010 között a fent megnevezett Doktori Iskola „A bioreguláció molekuláris és élettani szerveződése” programjának keretében irányításommal végezte munkáját. Az értekezésben foglalt eredményekhez a jelölt önálló alkotó tevékenységével meghatározóan hozzájárult. Az értekezés elfogadását javasolom.

Debrecen, 2010 . . . . . .

a témavezető aláírása

A vese apoptótikus endonukleázainak szerepe a bejuttatott idegen DNS/RNS lebontásában

Értekezés a doktori (Ph.D.) fokozat megszerzése érdekében
a MOLEKULÁRIS SEJTBIOLÓGIA tudományágban

Írta: **Buzder Tímea** okleveles biológus/genetikus

Készült a Debreceni Egyetem Juhász Nagy Pál Doktori Iskola keretében

Témavezető: DR. BÁNFALVI GÁSPÁR

A doktori szigorlati bizottság:
elnök: Dr.
tagok: Dr.
Dr.
A doktori szigorlat időpontja: 200… . …… … .

Az értekezés bírálói:
Dr.
Dr.
Dr.

A bírálóbizottság:
elnök: Dr.
tagok: Dr.
Dr.
Dr.
Dr.

Az értekezés védésének időpontja: 201… . …… … .

RÖVIDÍTÉSEK

Bicinkoninsav	BCA
cián fluoreszcens protein	CFP
deoxyribonukleáz I	DNaseI
dupla szálú	ds
dupla szálú törések	DSBs
egyszálú törések	SSBs
egyszálú DNS	ssDNA
endonukleáz G	EndoG
fluorescein isothiociánate	FITC
idegen DNS (foreign DNA)	fDNS
in situ nick transzláció	ISNT
knockout	KO
plasmid enhanced cyan fluorescent protein	pECFP-N1
plasmid incision assay	PIA
primer tubuláris vese epitél (primary tubular epithelial) sejt	PTE
RNS interferencia	RNAi
rövid hairpin RNS	shRNA
rövid interferáló RNS	siRNA
single radial enzyme diffusion assay	SRED assay
TdT-mediated dUTP nick end jelölés	TUNEL
vese tubuláris epitél (kidney proximal tubular epithelial) sejt	TKPTS
wild-type	WT

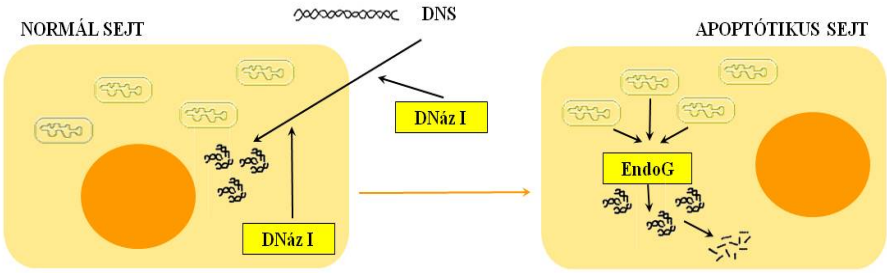
I. BEVEZETÉS

A bejuttatott DNS lebomlása napjainkban a génterápiák alapvető problémáját okozza. A cytotoxikus/apoptótikus endonukleázok arról ismeretesek, hogy megemésztik az "idegen DNS/RNS-t". Azonban, hogy melyek ezek az endonukleázok és ezen enzimek inaktiválásával növelhető-e a génbevitel sikeressége korábban nem képezte vizsgálat tárgyát, mivel endonukleáz-deficiens (KO- knock out) egerek, valamint endonukleáz gátlók nem álltak rendelkezésre.

Kutatásaim során - amit Arkansas állam orvosi egyetemének nephrológiai osztályán végeztem (University of Arkansas for Medical Sciences, Department of Internal Medicine, Division of Nephrology) - azt találtam, hogy a deoxyribonukleáz I (DNáz I) és az endonukleáz G (EndoG) a vese tubuláris epitél sejtekben (TKPTS- mouse kidney proximal tubule epithelial cell) jelen lévő legfőbb nukleinsav bontó enzimek.

Vizsgálataim alkalmával immortalizált TKPTS, valamint DNáz I és EndoG knockout (KO) egerekből izolált primer vese tubuláris epitél sejteket (PTE- primary tubular epithelial cells) használtam. A sejtekbe transzfekcióval bejuttatott pECFP-N1 plazmid vagy fluoreszcens siRNS celluláris endonukleázok révén történő lebontását vizsgáltam. Annak megállapítása céljából, hogy az intra, - vagy az extracelluláris DNáz I tölt be nagyobb szerepet a bejuttatott DNS lebontásában G-aktint használtam, valamint specifikus apoptózisgátlók hatását vizsgáltam a génbevitel hatékonysága szempontjából. A sejtbe jutatott idegen DNS lebontásának sematikus rajzát az 1. ábra bal oldali panelje mutatja be. Ebben a

folyamatban a fő szerepet játszik a sejtek legfontosabb DNS bontó enzime, a DNáz I. Apoptótikus sejtekben valószínűleg a mitokondriális eredetű EndoG nukleáz bontja le a sejtmagból kiszabaduló saját DNS-t is (1. ábra jobb oldali panel).



1. ábra. A csupasz DNS DNáz I és EndoG általi lebontásának feltételezett sémája

Disszertációmban a következő kérdések megválaszolását tűztem ki célul:

1. DNáz I és EndoG inaktiválják-e a vese tubuláris epitheliális sejtekbe transzfekció során bejuttatott DNS-t?

2. Ez a két endonukleáz együttműködik-e? Endonukleolitikus hatásuk az idegen DNS lebontására megfelel-e az 1. ábrán vázolt sémának?

3. Növelhető-e a gén bejuttatás mértéke ezeknek az enzimeknek a gátlásával?

A kérdések megválaszolása a jövőben segítséget nyújthat sikeres génterápiás stratégia kidolgozásához.

II. IRODALMI ÁTTEKINTÉS

Napjainkban a génterápiák „Achilles-sarkát" a gén célbajuttatása jelenti. A transzfekció számos típusa ismeretes attól függően, hogy mit próbálunk bejuttatni, milyen módszerrel és milyen módon (*in vivo/in vitro*), de általánosan megállapítható, hogy egyik sem ért el átütő sikert, aminek oka a DNázok szerepében kereshető. A DNázok az örökítőanyag lebontásáért felelős enzimek, széles körben ismert szubsztrát specifitásuk, kémiai mechanizmusuk és biológiai funkciójuk alapján.

1. Génbevitel

A sejtek extracelluláris DNS felvétele rendszerint bekövetkezik normál szövetfejlődés (Bergsmedh és mtsai., 2006; Yan és mtsai., 2006), vírus,- vagy baktériumfertőzés során (Chu és mtsai., 2006; Metifiot és mtsai., 2007), valamint kísérleti állatok és sejttenyészetek genetikai manipulációja alkalmával (Freitas és mtsai., 2007; Glasspool-Malone és mtsai., 2002; Tanswell és mtsai., 1998). Az „idegen" (foreign) DNS (fDNS) bejutása a gazdasejt halálához vezethet (Li és mtsai., 1999) DNS-dependens sejthalált okozva. A transzfekció előtti DNázzal való kezelés ennek megelőzéséhez vezet (Stacey és mtsai., 1993). Az idegen DNS bejutásának mértékét az azt lebontó enzimek készlete korlátozza (Glasspool-Malone és mtsai., 2002; Tanswell és mtsai., 1998). Annak ellenére, hogy a DNS bejutás lehetősége növelhető annak módosításával, lipidrészecskébe/vírusba történő csomagolásával, a bejuttatott DNS védelme elégtelen (Freitas és mtsai., 2007; Glasspool-Malone és mtsai.,

2002; Tanswell és mtsai., 1998). A DNázok játszák a főszerepet a fDNS lebontásában még mielőtt a sejtekbe kerül, de azt követően is. Az ebben a folyamatban fő szerepet játszó két DNáz vizsgálata képezi kutatásunk tárgyát.

2. Vese endonukleázok

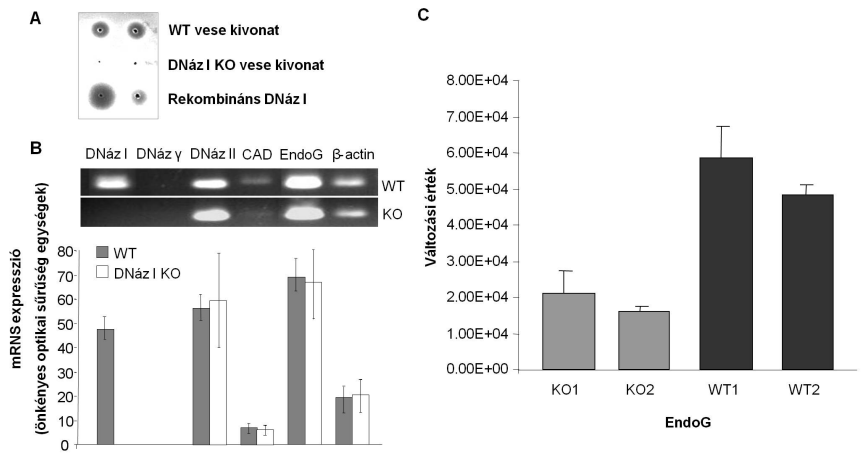
Korábbi vizsgálatok során megállapítottuk, hogy az egerek vese tubuláris sejtjeiben talált kilenc endonukleáz közül melyik képes a módosítás nélküli DNS nem-specifikus lebontására. Tisztázódott az is, hogy a deoxyribonukleáz I (DNáz I) a legaktívabb endonukleáz, az endonukleáz G (EndoG) pedig a második legaktívabb és a legnagyobb mértékben jelen lévő endonukleáz a vese tubuláris epitél sejtekben. Ez a két pro-apoptótikus endonukleáz felelős az egér vese teljes endonukleáz aktivitásának több mint 90%-ért (Basnakian és mtsai., 2005; Irvine és mtsai., 2005; Peitsch és mtsai., 1995). A DNáz I egy 31 kDa nagyságú citoplazmában jelen lévő enzim, amely a sejtből kiválasztódik, az egyszálú és a dupla-szálú DNS lebontásában van szerepe.

Az EndoG a celluláris DNS által kódolt, de a mitokondriumban lokalizálódó specifikus endonukleáz, mely az apoptózis során a mitokondriumból kiszabadul és a sejtmagba helyeződik át (Zhang és mtsai., 2003). 33 kDa nagyságú prekurzora a citoplazmában szintetizálódik, majd egy 28 kDa-os formává rövidülve lép a mitokondriumba (Ikeda és mtsai., 2001). Nukleázként szimpla-, és dupla szálú DNS, RNS, valamint DNA/RNA heteroduplexek lebontására is képes (Huang és mtsai., 2006). A DNáz I maximális aktivitása Ca^{2+}, Mg^{2+} ionok együttes jelenlétével érhető el (Basnakian és mtsai., 2002), míg az EndoG Mn-dependens enzim (Widlak és mtsai., 2001). A DNáz I AT-

gazdag szekvenciára specifikus endonukleáz, míg az EndoG GC-gazdag szekvencia specifitású. Ebből adódóan ezeknek az endonukleázoknak a széleskörű együttműködése tételezhető fel a lebontó folyamatokban (Widlak és mtsai., 2001).

3. Az endonukleáz hiányos sejtmodell

Sejtmodellként primer tubuláris epitél (PTE) sejteket használtunk, amelyek a frissen izolált tubuláris epitéllel megegyező endonukleáz fenotípussal rendelkeznek (Basnakian és mtsai., 2005). Az elsődleges sejtkultúrák arról ismeretesek, hogy nagymértékben rezisztensek DNS transzfekcióval szemben, ezért a DNS stabilitás javítása érdekében Lipofektamin-t használtunk PTE sejtek esetén. A DNáz I és az EndoG szerepének meghatározásához endonukleáz-deficiens egerekből izolált PTE sejteken végeztünk transzfekciós kísérleteket. Az *2. ábrán* látható, hogy az endonukleáz deficiencia homozigóta DNáz I KO egerek esetén teljes, míg heterozigóta EndoG egereknél részleges tulajdonságkiesés érhető el (Yin és mtsai., 2007).



2. ábra. A DNáz I teljes inaktiválása homozigóta DNáz I KO egerekben, valamint az EndoG részleges inaktiválása heterozigóta KO egerekben. **A**. *A SRED assay* (*single radial enzyme diffusion assay) azt mutatja, hogy a WT egerek veséjéből izolált proteinek tartalmazzák az aktív DNáz I-et, míg ez hiányzik a KO vese izolátumokból. Pozitív kontrollként humán rekombináns DNáz I-et (Dornase, Genentech) használtunk.* **B**. *WT- és DNáz I KO egerek veséjében expresszálódó "sejthalál-endonukleázok" semi-quantitatív RT-PCR vizsgálata és a keletkezett termék denzitometriás mérése megerősíti a DNáz I aktivitás teljes (95-100%) kiesését homozigóta DNáz I egerek esetén (Yin et al., 2007), valamint heterozigóta EndoG KO egereken 60-70%-os az aktivitás kiesés (***C***).*

4. Az endonukleázok szerepe az apoptózisban

Az idegen DNS-t elsősorban azok az endonukleázok támadják meg, amelyek elkülönítettek, így szabadon nem érhetőek el a citoplazmában. Az EndoG főként a mitokondriumban lokalizálódik, míg a DNáz I az endoplazmás retikulumban található. Ebből adódóan logikus az a feltevés, hogy vagy elegendő a jelen lévő, szabad citoplazmatikus EndoG és DNáz I aktivitása ahhoz, hogy a belépő DNS-t lebontsa, vagy a belépő

fDNS indukálja az endonukleázok kiszabadulását a sejt különböző kompartmentjeiből. Ez utóbbi indukciós folyamat részét képezheti a gazdasejt apoptózisának. Bár az idegen DNS apoptózist és DNáz enzimeket indukáló hatását leírták (Nur és mtsai., 2003; Stacey és mtsai., 1993), az apoptózis gátlását nem használták fel korábban a génbevitel hatékonyságának növelésére. A mi kísérleteink éppen ez utóbbi lehetőség kiaknázását hivatottak előmozdítani.

5. Apoptózis-inhibitorokban rejlő kísérleti lehetőségek

A sejthalálnak két formája különíthető el, a nekrózis és az apoptózis. Az apoptózis, más néven programozott sejthalál az eukarióta sejtek pusztulásának leggyakoribb formája. Ennek intrinszik útvonala egy fiziológiás öngyilkos mechanizmus, amely a homeosztázis fenntartására irányul és a szöveti megujulás természetes folyamata (Wyllie és mtsai., 1980). Az apoptózist elszenvedő sejtek a sejtmag és a citoplazma szerkezeti változásainak jellegzetes mintázatát mutatják, beleértve a plazmamemrán gyors kidudorodását (blebbing, a cytoskeleton feldarabolódik és a membrán kitüremkedését okozza) és a sejtmag szétesését. Ez utóbbi összefügg a kromatin nagymértékű sérülésével és a DNS hasításával a calcium-dependens endonukleázok aktiválását követően (Compton, 1992).

Az idegen DNS által indukált apoptózis a DNS sérülés intrinszik, mitokondriális útvonalát használja, melynek kulcs molekulái a caspase-2, 3, 9 és a p53 protein, Erre alapozva különböző apoptózis inhibitorokat teszteltünk: az endonukleáz inhibitor aurintrikarbonsav-ot (ATA), a caspase-3 inhibitor cink-kloridot ($ZnCl_2$), az antioxidáns cink-N-acetilciszteint (Zn-NAC), a p53 inhibitor pifithrin-t (PFT) és a pan-

caspase inhibitor benzoilkarbonil-Val-Ala-Asp-fluorometilketon-t (Z-VAD-fmk).

III. ANYAGOK ÉS MÓDSZEREK

1. Kísérleti állatok

A DNase I homozigóta knockout (DNase I KO) egereket (CD-1 background) Dr. T. Moroy-tól kaptuk (University of Essen, Németország), az EndoG knockout egerek Dr. M. Xu és Dr. J. Zhang-tól származtak (University of Cincinnati, OH). Mivel az EndoG -/- homozigóta egerek nem életképesek ezért, a sejteket heterozigóta egerekből izoláltuk (EndoG KO). Az összes egeret polimeráz láncreakcióval (PCR) genotipizáltuk (Djurovic és mtsai., 2004; Zhang és mtsai., 2003). Állatkísérleteinket a „Laboratóriumi Állatok Gondozása és Használata" (Guide for the Care and Use of Laboratory Animals, National Academy of Sciences) segédletben leírtaknak megfelelően hajtottuk végre, melyeket az Animal Care and Use Committee of the Central Arkansas Veterans Healthcare System hagyott jóvá.

2. Sejtkultúrák

Az elsődleges egér vese tubuláris epitél sejteket (PTE) frissen izoláltuk DNáz I KO, EndoG KO, valamint vad típusú (WT) egerekből (Nowak és mtsai., 2003) és kísérleteinket megelőzően 10 napig tenyésztettük.

Az immortalizált egér vese tubuláris epitél sejteket (TKPTS) Dr. Elsa Bello-Reuss-tól kaptuk (University of Texas Medical Branch, Galveston, TX). Ezeket a korábban leírt módon tenyésztettük (Ernest és mtsai., 1995) 7% FBS-el (fetal bovine serum) kiegészített (Hyclone, Logan, UT) DMEM/HAM F-12 médiumban (Sigma-Aldrich Co. St.Louis, MO). A tenyészeteket CO_2 inkubátorban, 37 °C-on, 5% CO_2-ban tartottuk, 48-72

órás időközönként etettük és a konfluencia elérését követően 1 napon belül felhasználtuk.

3. Sejtkultúrákból teljes fehérje izolálás

A sejttenyésztést követően a sejteket centrifugálással összegyűjtöttük (1500rpm, 3 min., 4 ºC), a felülúszó eltávolítása után a sejteket PBS-ben (phosphate buffered saline) felszuszpendáltuk, majd újra lecentrifugáltuk (1500rpm, 3 min., 4 ºC).

A fehérjék kinyerése céljából a sejteket szuszpendáltuk 100µl Puffer A-ban (50mM Tris-HCl, pH7.9; 0.25M szacharóz, Komplett Mini Proteináz Inhibitor Cocktail, (Roche Diagnostics, Mannheim, Németország) (1 tabletta/10 ml)) és 2 x 20 másodpercig ultrahanggal kezeltük (Virsonic 475, Virtis, Gardiner, NY). A makrorészecskéket kicsaptuk magas fordulatszámon centrifugáltuk (14000 rpm, 10 min., 4 ºC) majd a felülúszót összegyűjtöttük. A protein kivonat tároló pufferben (55% glicerin, 10mM Tris-HCl pH 7.6, 0.5mM dithiotreitol) szemben dializálva −20°C-on, 2 hétig eltartható endonukleáz-aktivitás vesztés nélkül.

A fehérjekoncentrációt bicinkoninsav (BCA) protein assay (Pierce, Rockford, IL) segítségével határoztuk meg, BSA-t (Bovine serum albumin) használva standardként.

4. Plazmid hasítás vizsgálata

A vesesejtekből és tenyészmédiumból izolált teljes protein aktivitását plazmid hasítással (plasmid incision assay, PIA) határoztuk meg.

pBR322-t (New England Biolabs, Beverly, MA) használtunk szubsztrátként (Basnakian AG, 2005).

Annak ellenőrzése céljából, hogy a Lipofektamin megvédi a plazmid DNS-t az endonukleázok által történő emésztéstől, a reakciót megelőzően a pBR322 plazmidot előkezeltük Lipifectaminnal. A plazmidot és a Lipofektamint külön szérummentes DMEM/HAM F-12 médiummal (Sigma-Aldrich) hígítottuk, majd összekevertük és 20 percig, szobahőmérsékleten inkubáltuk.

A sorozat hígítással készült mintákat (1, 1:5, 1:25, 1:125, 1:625) a reakcióelegyhez adtuk (1µg pBR322 plazmid DNS, 2mM $CaCl_2$, 5mM $MgCl_2$, 10mM Tris-HCl, pH 7.4 és 0.5mM dithiothreitol), a reakciót 1 órán át, 37 °C-on inkubáltuk, majd Stop-oldat (10mM Tris-HCl, pH 7.4, 1% SDS, 25mM Na_2EDTA, 7.5mM brómfenolkék) hozzáadásával állítottuk le. A mintákat 1%-os agaróz gélen, TAE (Tris-acetát-EDTA, pH 8,0) pufferben futtattuk (7 V/cm, 35 min.). A DNS-t ethidium bromid festéssel tettük láthatóvá. EagleEye szkennelő denzitométert (Stratagene, La Jolla, CA) használtunk az endonukleáz-kezelt plazmid DNS formák, így a kovalensen zárt körkörös (C, covalently closed circular DNA), a nyílt körkörös (O, open circular DNA) és a lineáris (L, linear DNA), valamint emésztett formájának (D, digested form) meghatározására. Egy egység az az endonukleáz mennyiség, amely 1 óra alatt, 37 °C-on, 1 µg kovalensen zárt szupertekercselt plazmid DNS-t nyílt körkörös lineáris vagy emésztett formába képes átalakítani.

PIA assay-t használtunk a primer sejtek endonukleázainak jellemzésére is. A fentiekben leírt módon mértük mintáink Ca^{2+}/Mg^{2+}-dependens endonukleáz (elsősorban DNázI) aktivitását, amelyek 2mM $CaCl_2$-ot, 5mM $MgCl_2$-ot, 10mM Tris-HCl-t, pH 7.4, 0.5mM dithiothreitol-t

tartalmaztak. A mangán-dependens endonukleáz G aktivitás meghatározáskor mintáink 5mM $MnCl_2$-ot, 10mM Tris-HCl-t, pH 7.4, 0.5mM dithiothreitol-t tartalmaztak.

5. Real-time RT-PCR

Saját, jól bevált protokollunkat használtuk (Basnakian és mtsai., 2006). SmartCycle PCR készülékben (Cepheid, Sunnyvale, CA) real-time RT-PCR-t követően 1µg teljes RNS-t reverztranszkripcióját hajtottuk végre. A reakcióelegyet Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) felhasználásával készítettük a gyártó javaslatai alapján. A primerek a következők voltak:

EndoG: 5'-GATGAGACCATCCCTCTGGA-3'
5'ATGTGAGTC AGCCCATCTCC-3'
DNáz I: 5'-ACTCAATCGGGACAAACCTG-3'
5'-ATTTCCACA GGGTTCACAGC-3'

A relatív RNS koncentrációjának meghatározására a Cepheid SmartCycle szoftvert (Version 2.0d) használtuk.

6. Plazmid transzfekció

A pECFP-N1 plazmid a cián fluorescein proteint (CFP) kódolja. A PTE sejteket pECFP-N1 plazmiddal transzfektáltuk (Clontech Laboratories, Inc., Mountain View, CA) Lipofektamin 2000 transzfekciós reagenssel (Invitrogen Co., Carlsbad, CA), a gyártó protokollja alapján. A sejteket 6 lyukú lemezekbe szélesztettük 24 órával a transzfekciót megelőzően. 4µg plazmid DNS-t és 1:7.5 DNS/liposzóma arányt hígítottunk külön csövekben, amelyek 250-250µl szérummentes DMEM/HAM F-12 médiumot (Sigma-Aldrich) tartalmaztak. Ezeket

összekevertük, majd 20 percig, szobahőmérsékleten inkubáltuk. A sejtekhez 2ml szérummentes DMEM/HAM F-12 médiumot (Sigma-Aldrich) adtunk, majd cseppenként folyamatos mozgatás közben a transzfekciós komplexet. 24-48 h ikubációt (37 °C, 5% CO_2) követően a CFP expresszióját fluoreszcens mikroszkóppal detektáltuk.

7. Kis interferáló RNS bevitele a sejtekbe (siRNS transzfekció)

A PTE sejteket TransIT-TKO transzfekciós reagens (Mirus Bio Co., Madison, WI) segítségével transzfektáltuk. A sejteket 6 lyukú lemezeken szélesztettük 24 órával a transzfekciót megelőzően. 18µl transzfekciós reagenst 250µl-re hígítottunk szérummentes DMEM/HAM F-12 médiummal (Sigma-Aldrich). Az elegyet 15 percig inkubáltuk, majd 75µl (1µM) siRNS/fluoreszcens siRNS-t (Label IT RNAi Delivery Control-Fluorescein, Mirus Bio Co., Madison, WI) adtunk hozzá és további 20 percig inkubáltuk szobahőmérsékleten. A sejtekhez 1172µl szérummentes DMEM/HAM F-12 médiumot (Sigma-Aldrich) adtunk, majd cseppenként a transzfekciós komplexet. Két napos inkubálást (37 °C, 5% CO_2) követően a fluoreszcens jelzést hordozó siRNS expresszióját fluoreszcens mikroszkóppal detektáltuk.

A Label IT RNAi Delivery Control – Fluorescein, kémiai festékanyag, mely Fluorescein Izotiocianátot (FITC) tartalmaz. A FITC jelölőanyag kapcsolómolekulán keresztül kovalensen kötődik a nukleotidokhoz. Rövid duplaszálú RNS-ek (Wyllie és mtsai.) más néven kis interferáló RNS-ek (small interfering RNA, siRNA) emlős sejtekbe történő bejuttatása célzott mRNS szekvenciák specifikus gátlását okozza. Ez a mRNS-ek által kódolt fehérjék expressziójának csökkenéséhez vezet. Az RNS interferencia (RNAi) hatása több sejtosztódást követően is

detektálható lehet. Az siRNS-nek ezek a tulajdonságai teszik rendkívül hatékonnyá a célszekvenciák expressziójának gátlásában. A Label IT RNAi Delivery Control szekvenciája nem homológ egyetlen ismert emlős génnel sem és nincs tudomásunk róla, hogy befolyásolná a sejtben végbemenő folyamatokat. Úgy tervezték, hogy az *in vivo* vagy *in vitro* RNAi kísérletek során optimális legyen a dsRNS oligonukleotidok bejutása és láthatóvá tétele (Mirus Bio Co., Madison, WI, Lit.# ML039).

A TKPTS sejteket TransIT-TKO transzfekciós reagens (Mirus Bio Co., Madison, WI) segítségével transzfektáltuk. A sejteket 24 lyukú lemezekre szélesztettük 24 órával a transzfekciót megelőzően. 4µl transzfekciós reagenst 50µl-re hígítottunk szérummentes DMEM/HAM F-12 médiummal (Sigma-Aldrich) és 15 percig inkubáltuk. Ezután 15µl (1µM) siRNS-t adtunk hozzá és további 20 percig inkubáltuk szobahőmérsékleten. Ezt követően 250µl szérummentes DMEM/HAM F-12 médiumot (Sigma-Aldrich), majd cseppenként a transzfekciós komplexet adtunk a sejtekhez. Két órás inkubálást (37 °C, 5% CO_2) követően a médiumot szérummentesre cseréltük, majd további 46 h inkubálás után a sejteket pECFP-N1 plazmiddal transzfektáltuk (lásd Plazmid transzfekció, Anyagok és módszerek). 24-48h inkubálást (37 °C, 5% CO_2) követően a CFP expressziót fluoreszcens mikroszkóppal detektáltuk.

EndoG siRNA target szekvencia: AAAUGCCUGGAACAACCUUGA (Dharmacon, Lafayette, CO)

DNáz I siRNA target szekvencia: TGACATCGCTGTTATCCAA (Dharmacon, Lafayette, CO)

siCONTROL Non-Targeting siRNA (Dharmacon, Lafayette, CO)

8. Apoptózis gátlás hatása a transzfekció hatékonyságára

TKPTS sejteket cink-klorid ($Zn\text{-}Cl_2$), aurintrikarbonsav (ATA), zinc N-acetylcysteine (Zn-NAC), pifithrin (PFT) és benzoilkarbonil-Val-Ala-Asp-fluorometilketon (Z-VAD-fmk) apoptózis inhibitorokkal kezeltük a pECFP-N1 a plazmid transzfekcióját megelőzően. A cink-vegyületeket kollaboráló partnerünktől, Dr. Richard B. Walker-től (Department of Chemistry and Physics, University of Arkansas) kaptuk. A sejteket 6 lyukú plate-ekben 5µl különböző koncentrációjú gátlószerekkel kezeltük és médiummal 500 µl-re egészítettük ki az optimális koncentráció megállapítása érdekében, majd 30 perc inkubálást követően transzfektáltuk a Plazmid transzfekció fejezetben leírtak szerint. Éjszakán át inkubáltuk (37 °C, 5% CO_2), majd a CFP expresszióját fluoreszcens mikroszkóppal detektáltuk.

9. TUNEL assay

A TKPTS sejteket 6 lyukú platek-be helyezett steril fedőlemezekre szélesztettük, majd apoptózis inhibitorokkal kezeltük és pECFP-N1 plazmiddal transzfektáltuk a fent leírtak szerint. A sejteket 28 órán át inkubáltuk (37 °C, 5% CO_2) a TUNEL assay-t megelőzően (Karan és mtsai., 2004; Yang és mtsai., 2006).

A sejtekről eltávolítottuk a médiumot, PBS-el mostuk, majd 4%-os paraformaldehid (PFA) oldattal fixáltuk. Az assay-t az *in situ* sejthalál detektáló kit (*In Situ* Cell Death Detection Kit, Roche Diagnostics, Indianapolis, IN) segítségével végeztük el, mely a fixált sejtken kívül terminális deoxinukleotid transzferázt (TdT), anti-aktin-FITC prekurzort tartalmazott kakodilát pufferben. Az 1 órás, 37°C-on történő inkubálást követően a sejteket háromszor mostuk PBS-ben. A lemezeket végül

DAPI tartalmú ProLong Mounting oldattal (Molecular Probes) fedtük le és 3-24 h sötét helyen tartottuk, majd fluoreszcens mikroszkóppal detektáltuk.

Az *in situ* sejthalál detektáló fluoreszcens kit egyszálú és kétszálú töréseket ismer fel, amelyek az apoptózis korai szakaszában keletkeznek. Ha a fixált és permeabilizált apoptótikus sejteket inkubáljuk a TUNEL reakcióeleggyel, amely TdT-t (terminális deoxinukleotid transzferáz) és fluoreszcein-dUTP-t (deoxiuridin trifoszfát) tartalmaz, a TdT enzim katalizálja a DNS egy,-és kétszálú szabad 3'-OH végein a fluoreszcein-dUTP beépülését. A fluoreszcens jel a DNS sérült, szabad részeihez kapcsolódik, és áramlásos citometriával, valamint fluoreszcens mikroszkóppal kimutatható.

10. Statisztikai analízis

A statisztikai analíziseket a Two-way ANOVA és a Student's teszttel végeztük el. Az eredményeket az átlag ± átlag szórása (mean ± standard error of mean, SEM) értékekkel határoztuk meg, a $P < 0.05$-öt tekintettük szignifikánsnak.

IV. EREDMÉNYEK

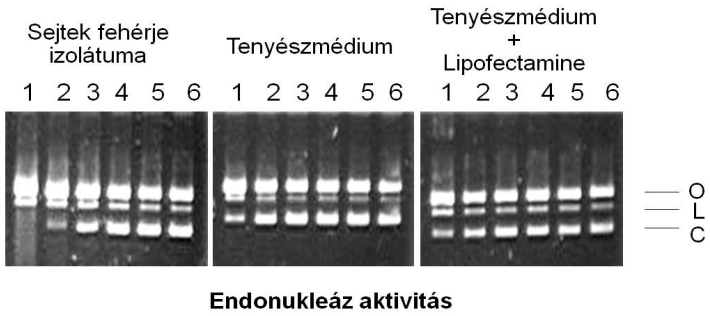
1. TKPTS sejtek endonukleáz aktivitása

Kísérletünk célja az volt, hogy megállapítsuk, hogy a plazmid DNS emészthető-e a vese tubuláris epitél sejtekben (TKPTS) lévő, valamint a tenyészmédiumba kiválasztott endonukleázokkal. A tápfolyadékban növesztett konfluens sejteket külön-külön összegyűjtöttük. Teljes (total) proteint izoláltunk belőlük az Anyagok és módszerekben leírtak szerint, majd meghatároztuk fehérje koncentrációjukat. Endonukleáz aktivitásuk mérését plazmid hasításos assay-el hajtottuk végre.

A pBR322 plazmid DNS-ek jelzése: kovalensen zárt, törést, hasítást nem tartalmazó, cirkuláris, szupertekercselt DNS (C, cirkuláris), az egyszálú (single-stranded breaks, SSBs) törésekkel relaxált, nyílt, de még körkörös DNS (O, open), és a kétszálú lánctörést (DSB) is tartalmazó lineáris DNS (L, lineáris) *(3.ábra)*. Az endonukleáz aktivitás magasabb volt a fehérje kivonatban, mint a külső médiumban, amiből arra következtethetünk, hogy a plazmid DNA lebomlása főként a sejtek belsejében történik. A legkompaktabb a kovalensen zárt, cirkuláris, szupertekercselt DNS (C), ezért ennek a formának a legnagyobb az elektroforetikus mobilitása az agaróz gélben. Az elektroforézis során ez kerül legtávolabb a start ponttól. Ezt követi a lánctörést tartalmazó és ezzel lineárissá váló lineáris plazmid (L) mobilitása, míg legkisebb az egyszálú hasítást tartalmazó, nyílt (O), de még körkörös plazmid DNS elektroforetikus mozgékonysága *(3. ábra)*.

A csupasz plazmid DNS emlős sejtekbe juttatása (transzfekciója) Lipofektaminnal tehető hatékonyabbá. A Lipofektamin egy transzfekciós

reagens, melyet siRNS vagy plazmid DNS lipofekciós beviteléhez használnak *in vitro* sejtkultúrákban. Annak ellenőrzése céljából, hogy a Lipofektamin-nak van-e védő funkciója az endonukleázos DNS bontás ellen, a pBR322 plazmidot előkezeltünk ezzel a transzfekciós reagenssel (Djurovic és mtsai., 2004). Amint a *3. ábra* mutatja a Lipofektamin önmagában nem nyújt védelmet a plazmid DNS-nek a tápfolyadékban lévő *in vitro* endonukleázos bontásával szemben.

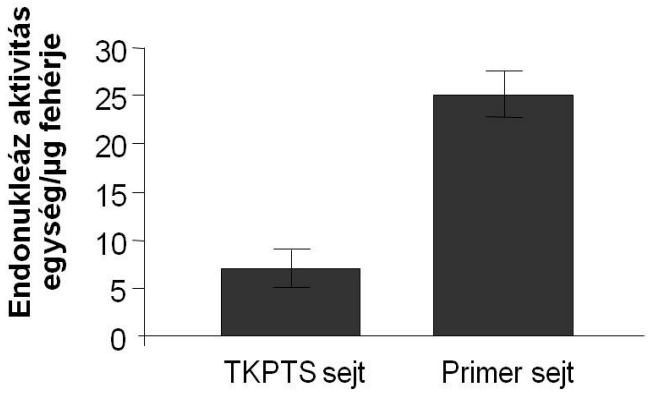


3. ábra. A TKPTS sejt endonukleáz aktivitása. *A sejt extrakt/médium hígítási sora (1-6): 1:1, 1:5, 1:25, 1:125, 1:625, és 1:3125. O- nyílt kör alakú DNS; L- lineáris DNS; C- kovalensen zárt DNS*

2. Az immortalizált és primer sejtek endonukleáz aktivitásának összehasonlítása

A primer sejtekről ismeretes, hogy DNS transzfekcióval szemben ellenállóak (Stacey és mtsai., 1993; Welter és mtsai., 2004; Zhong és mtsai., 2005). Annak érdekében, hogy kiderítsük, hogy az "idegen DNS" elleni rezisztencia összefügg-e a primer sejtek magas endonukleáz aktivitásával, összehasonlítottuk az immortalizált TKPTS, valamint a primer PTE sejtekből izolált proteinek teljes endonukleáz aktivitását.

A plazmid hasítás vizsgálata a *4.ábrán* azt mutatja, hogy az endonukleáz aktivitás primer sejtekben sokszorosa az immortalizált sejtekben lévő DNS bontásnak. Az endonukleáz aktivitás immortalizált sejtekben 25±2 egység/µg protein PTE sejtekben, míg TKPTS sejtekben csak 7±3 egység/µg fehérje. Ez a megfigyelés alátámasztja azt az elképzelésünket, hogy a primer sejtek rezisztenciája nagymértékben nukleáz aktivitásuknak tulajdonítható feltételezve, hogy a primer és immortalizált sejtek membránpermeabilitása azonos.



4.ábra. Primer,-és immortalizált sejtek endonukleáz aktivitásának mérése. *pBR322 plazmid hasítás (plasmid incision assay, PIA)* Ca^{2+} *és* Mg^{2+} *ionok (2mM* $CaCl_2$*, 5mM* $MgCl_2$*) jelenlétében.*

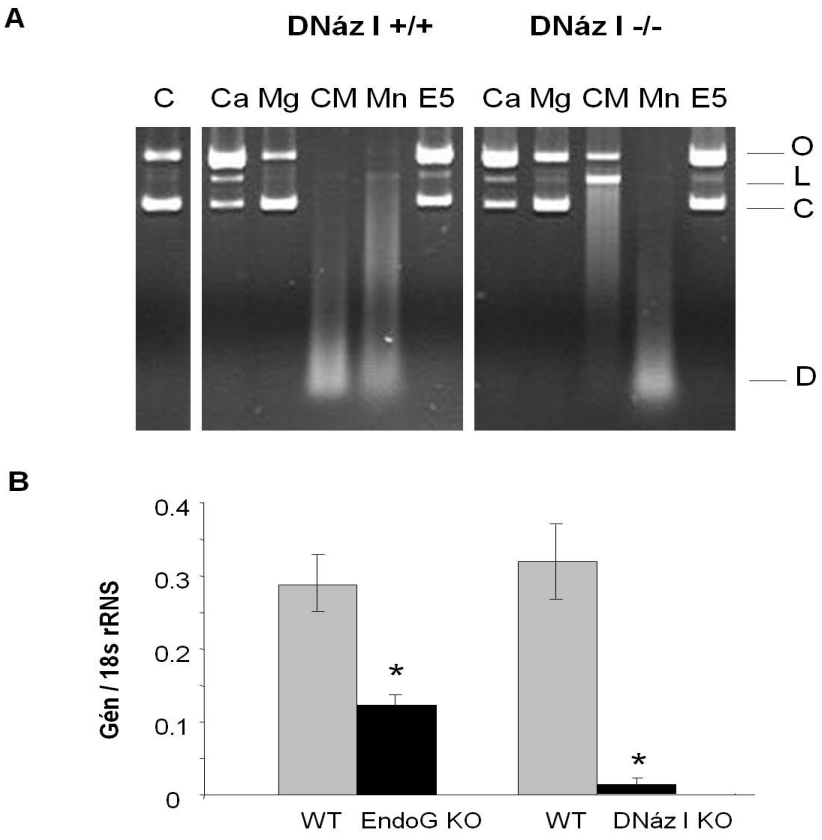
3. Endonukleázok jellemzése PTE sejtekben

A különböző endonukleázok átfedő kation és pH követelményekkel rendelkeznek, így összehasonlításuk nehézségekbe ütközött az egyes endonukleázokra hiányos (knockout, KO) egerek megjelenéséig.

Korábbi kutatásunk során a DNáz I és az EndoG bizonyult a két legaktívabb endonukleáznak rágcsálók veséjében (Basnakian és mtsai., 2005; Yin és mtsai., 2007). PTE sejteket izoláltunk (vad típusú) WT és KO egerekből, és az izolált proteineket plazmid hasítással (PIA) teszteltük különböző kationok jelenlétében.

Az eredmények azt mutatják, hogy a vad típusú egerek endonukleáz aktivitásának nagyobb része Ca/Mg-dependens, tehát a DNáz I a fő endonukleáz ezekben a sejtekben (*5.A. ábra*). A DNáz I inaktiválását követően (DNáz I KO egerekből izolált sejtek) a Mn-dependens endonukleáz a legaktívabb, így az EndoG-t tulajdonítjuk a második legaktívabb vesében lévő endonukleáznak (Widlak és mtsai., 2001). Az EndoG részleges inaktiválásával (heterozigóta EndoG KO egerekből izolált sejtek) csökkent Mn-dependens endonukleáz aktivitás is társul, de ez nincs hatással a Ca/Mg-dependens DNáz I aktivitásra (*ábrán nincs feltüntetve*).

Real-time RT-PCR-al megállapítottuk, hogy ezen endonukleázok expressziója milyen mértékben csökken KO sejtekben. A kapott adatok szerint a homozigóta DNáz I KO egerek esetén a DNáz I aktivitás teljes kieséséről (95-100%) beszélhetünk és a heterozigóta EndoG 60-70 %-os kieséséről EndoG KO egérvese esetén (*5.B. ábra*).



5.ábra. Endonukleázok aktivitása és kifejeződése primer tubuláris epitél sejtekben.

A. *függőleges sor: O- nyílt kör alakú DNS; L- lineáris DNS; C- kovalensen zárt DNS; D- lebontott DNS; vízszintes sor: kontroll nem-emésztett pBR322 DNS; Ca- 2mM $CaCl_2$, pH 7.5; Mg- 2mM $MgCl_2$, pH 7.5; CM- 2mM $CaCl_2$ + 2mM MgCl2; Mn- 2mM $MnCl_2$, pH 7.5; E5- 2mM EDTA, kationmentes, pH5 (DNase II aktivitás mérésére).*

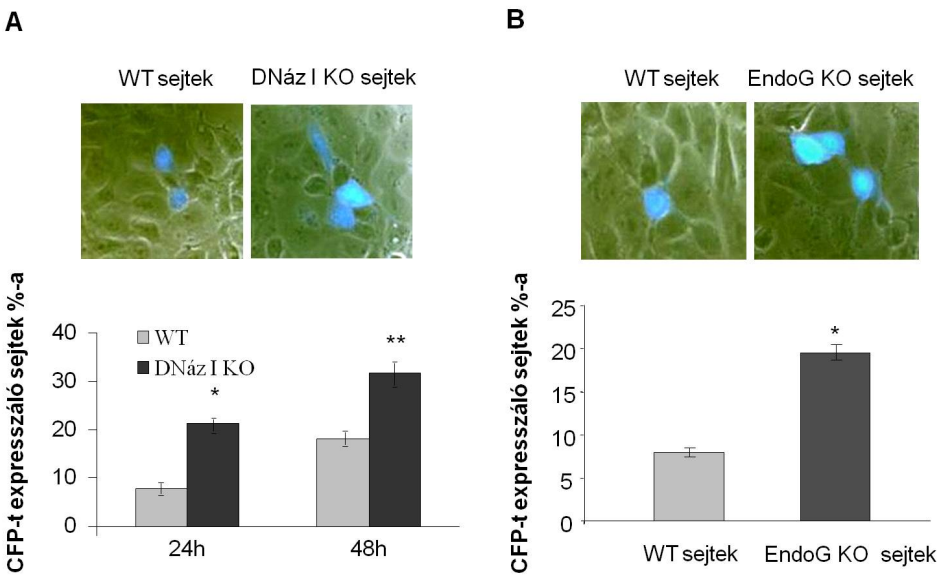
B. *n=4, *p<0.001*

4. Inaktivált DNáz I és csökkentett aktivitású EndoG szerepe a DNS transzfekcióban

A DNáz I inaktiválásával és az EndoG aktivitás csökkentésével befolyásolhatjuk a primer sejtekbe történő DNS transzfekciójának hatékonyságát. Hogy megvizsgáljuk ezt a lehetőséget cián fluoreszcens proteint (CFP) kódoló pECFP-N1 plazmiddal transzfektáltunk WT és KO sejteket. A *6.A. ábrán* látható, hogy két, 24 és 48 órás inkubálási időt használtunk transzfekciót követően. A CFP expressziója A DNáz I KO sejtekben szignifikánsan magasabb, mint a WT sejteben (24h: 21±5% vs. 8±5% transzfektált sejt, n=3, *$p<0.013$; 48h: 32±6% vs. 18±5% transzfektált sejt, n=3, **$p<0.025$).

Mivel 48 h inkubáláskor magasabb volt a sejtek transzfekciójának hatékonysága, így ezt választottuk a *6.B. ábrán* bemutatott kísérlethez, amikor WT és EndoG KO sejteket transzfektáltunk ugyanazzal a plazmiddal. A CFP expressziója a pECFP plazmid transzfekcióját követően EndoG KO sejtekben szignifikánsan magasabb, mint a WT sejtekben. (19±5% vs. 8±5% CFP-pozitív sejtek, n=6, *$p<0.001$).

Ezek az eredmények az bizonyítják, hogy az endonukleázok jelenléte csökkenti, hiánya pedig növeli a DNS transzfekció hatékonyságát.



6. ábra. A primer sejtek plazmid transzfekciójának hatékonysága aktív/inaktív endonukleázok jelenlétében.

5. Az RNS interferencia transzfekcióra gyakorolt hatása

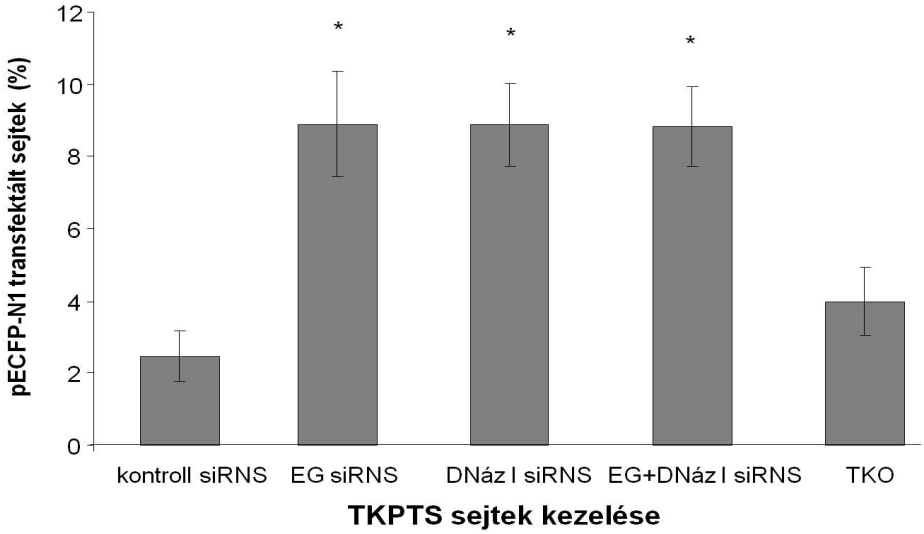
Az endonukleáz inaktiválás transzfekciót fokozó hatásának igazolására siRNS-eket használtunk ezen endonukleázok "csendesítésére" (silencing). TKPTS sejteket kezeltünk DNáz I-, EndoG vagy mindkét siRNS-el, majd pECFP-N1 plazmiddal transzfektáltuk.

EndoG siRNS target szekvencia: AAAUGCCUGGAACAACCUUGA

DNase I siRNA target szekvencia: GACATCGCTGTTATCCAA, (Dharmacon, Lafayette, CO). Az siRNS kezelt sejtek (DNáz I, EndoG vagy DNáz I+EndoG) szignifikánsan magasabb plazmid transzfekciót mutattak, mint az siRNS kontroll (8.8% vs. 2.4% of pECFP-N1

transzfektált sejtek, *p=0.001) vagy csak a transzfekciós reagenssel kezelt (TKO) kontroll sejtek (*7.ábra*).

Az eredmények bizonyítják, hogy az endonukleázok bármelyikének "csendesítése" jelentős mértékben növeli a tubuláris epitél sejtek transzfekciós hatékonyságát.



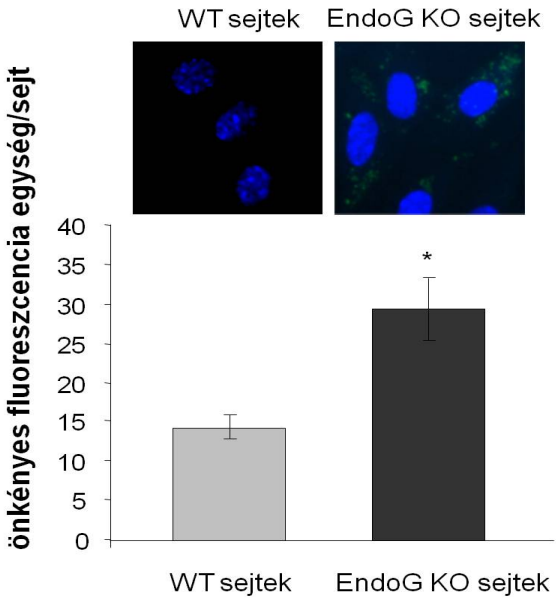
7. ábra. A plasmid transzfekció hatékonysága TKPTS sejtekben EG siRNS, DNáz I siRNS és EG+DNáz I siRNS kezelést követően. TKO- TransIT-TKO Transzfekciós Reagens; control siRNA- siCONTROL Non-Targeting siRNA.

6. Az EndoG inaktiválás RNS transzfekciót fokozó hatása

A DNáz I-el ellentétben az EndoG nukleáz mind a DNS, mind az RNS lebontására is képes (Kalinowska és mtsai., 2005). Következésképpen anti-DNS aktivitása mellett az EndoG fontos szerepet játszhat a sejtek RNS transzfekciójában is. Ennek kiderítésére EndoG KO és WT sejteket transzfektáltunk fluoreszcens-jelölt siRNS-el (Label IT RNAi Delivery

Control shRNA, Mirus Bio Co., Madison, WI). A Label IT RNAi Delivery Control fluoreszcensen-jelölt duplaszálú RNS duplexeket tartalmaz, melyek hosszúsága, töltése és konfigurációja az RNAi vizsgálatokban szereplő standard siRNS-ével megegyező.

A *8. ábra* bemutatja, hogy 48h inkubálást követően az EndoG KO sejtek transzfekciós rátája a WT sejtek kétszerese (14±2 vs. 29±4 önkényes fluoreszcencia egység/sejt, n=3, *p<0.01). A sejtmagot DAPI-val kék színűre festettük. Ez a megfigyelés arra utal, hogy az EndoG nagy valószínűséggel kettős szerepet játszik a gazdasejt védelmében az "idegen" DNS és RNS ellen.



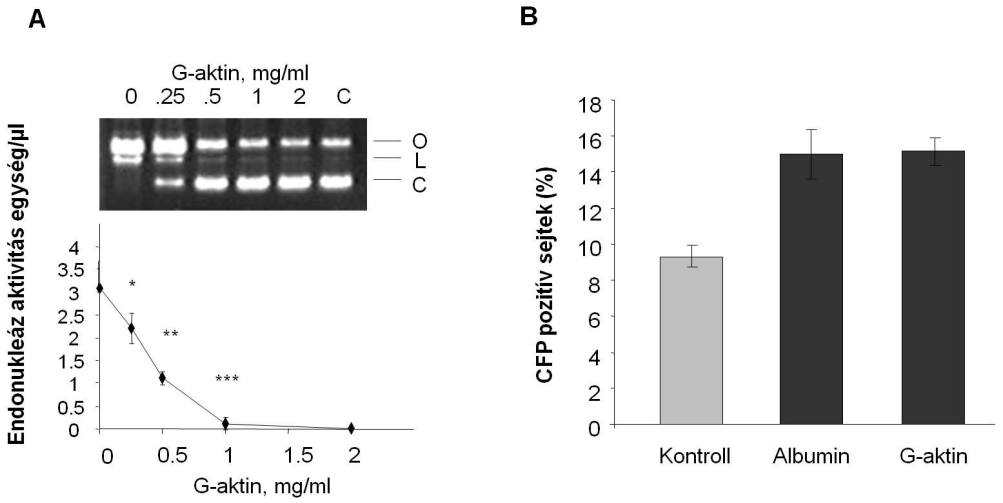
8. ábra. A primer sejtek RNS transzfekciójának hatékonysága aktív (WT) és inaktív (KO) EndoG jelenlétében

7. Extracelluláris DNáz I inaktiválás hatása a transzfekcióra

Míg az EndoG teljes mértékben intracelluláris enzim a DNáz I szekréciós enzim (Lacks, 1981). Az extracelluláris, szekterált DNáz I transzfekcióban betöltött szerepének meghatározása céljából G-aktin-t használtunk. A G-aktin a DNáz I specifikus és irreverzibilis inhibitoraként ismert (Lacks, 1981), nincs toxikus hatással a sejtekre, azokba nem lép be és így felhasználható arra, hogy az extracelluláris DNáz I-re hatva kiküszöbölje annak hatását.

TKPTS sejteket G-aktinnal kezeltünk 0 - 2 mg/ml koncentráció intervallumban. A kezelést követően az endonukleáz aktivitást plazmid hasítási teszttel határoztuk meg (*9.A. ábra*). Teljes aktivitás gátlást a G-aktin 1 mg/ml koncentrációjánál értünk el (n=4, *p=0.018, **p=0.0052, ***p=0.006).

Második kísérletünkben TKPTS sejteket kezeltünk 1 mg/ml G-aktinnal vagy kontrollként 1 mg/ml albuminnal, majd pECFP plazmiddal transzfektáltuk a sejteket (*9.B. ábra*). Nem találtunk különbséget a G-aktin és a kontroll albuminnal kezelt sejtek közötti transzfekciós hatékonyságban (15±5 vs. 15±8% CFP-pozitív sejt, n=6), amiből arra következtethetünk, hogy az extracelluláris DNáz I nincs hatással a transzfekcióra és nagy valószínűséggel nem vesz részt a DNS ellenes sejtvédekezésben.



9. ábra. Az extracelluláris DNáz I nincs hatással a DNS transzfekció hatékonyságára.

8. Génbevitel hatékonyságának növelése az apoptózis gátlásával

Irodalmi utalások történtek arról, hogy a bejuttatott, idegen DNS apoptózist indít el és a sejt endonukleázai aktiválódnak (Nur és mtsai., 2003; Stacey és mtsai., 1993), de az apoptózis gátlását nem kísérelték meg felhasználni a génbevitel hatékonyságának növelésére. Számos módszer ismert az apoptótikus sejtek azonosítására (Afanasyev és mtsai., 1993; Bryson és mtsai., 1994; Darzynkiewicz és mtsai., 1992).

Az endonukleolízis az apoptózis kulcsfontosságú biokémiai folyamata, ami a DNS oligonukleoszóma méretű feldarabolódását okozza. Ezt a folyamatot gyakran használják az apoptózis elektroforetikus detektálására. Ez az eljárás viszont nem szolgál információval az egyedi sejteket illetően, sem a sejtapoptózis szöveti lokalizálódásáról, sem a sejtdifferenciációval való összefüggéseiről. Az apoptózis ilyen jellegű vizsgálata a DNS szál törésének enzimatikus *in situ* jelölésével érhető el.

DNS polimeráz, valamint terminális nukleotid transzferáz (TdT) használatos a jelölt nukleotidok és a DNS szál törésének eredeti helyzetben történő összekapcsolására (Gold és mtsai., 1994; Gorczyca és mtsai., 1993; Sgonc és mtsai., 1994; Zager és mtsai., 1994).

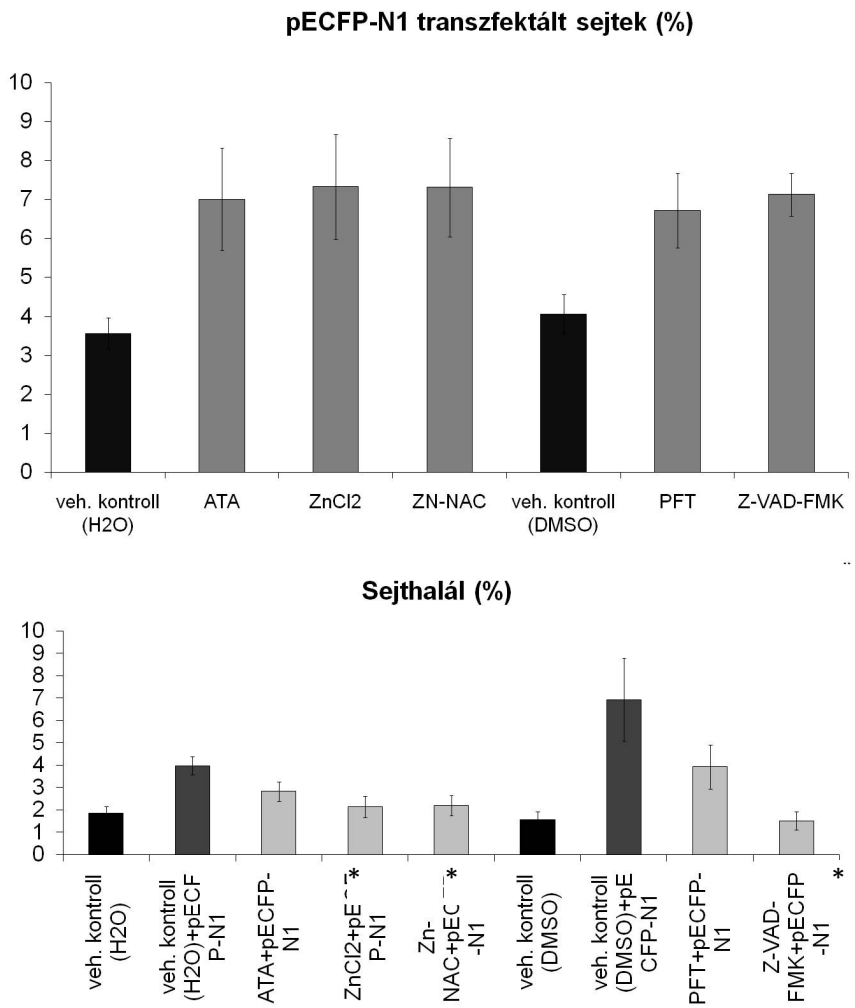
A "tailing" reakció, amit a TdT enzim katalizál TUNEL assay-ként (TdT-mediated dUTP nick end labeling) is ismeretes (Gavrieli és mtsai., 1992; Mochizuki és mtsai., 1994; Portera-Cailliau és mtsai., 1994; Sgonc és mtsai., 1994). Segítségével fluoreszcens jelzést hordozó nukleotidok építhetők a hasítást tartalmazó DNS-be. Jelentős előnye a DNS polimeráz által katalizált *in situ* nick transzlációval (ISNT) szemben, hogy nagyobb jelölési sűrűség érhető el, a reakció kinetikája gyorsabb, mint az ISNT esetén, valamint a TUNEL metodika specifikusabb az apoptótikus, minta a nekrotikus sejtekre, ezért az apoptózist elkülöníti a nekrózistól.

Kísérletünk során az apoptózis inhibitorainak alkalmazásával gátoltuk az idegen anyag által kiváltott apoptózist, amit TUNEL-assay-vel detektáltunk. Ezzel indirekt módon gátoltuk az érintett endonukleázok aktivitását, majd plazmid-transzfekciót követően vizsgáltuk a génbevitel hatékonyságát.

TKPTS sejteket aurintrikarbonsav (ATA), cink-klorid ($ZnCl_2$), zinc N-acetilcisztein (Zn-NAC), pifithrin (PFT) és benzoilkarbonil-Val-Ala-Asp-fluorometilketon (Z-VAD-fmk) apoptózis inhibitorokkal kezeltük pECFP-N1 plazmidtranszfekciót megelőzően (Anyagok és módszerek). Az inkubálást követően a CFP expresszióját fluoreszcens mikroszkóppal detektáltuk. A *10.A. ábrán* látható, hogy a kezelt sejtek a kontroll sejtekhez képest szignifikánsan magasabb, közel kétszeres transzfekciót mutatnak (ATA: 7±1.3%, Zn-Cl_2: 7.3±1.3%, Zn-NAC: 7.3±1.3% *versus* H_2O kontroll: 3.6±0.5%; PFT: 6.7±0.9%, Z-VAD-fmk: 7.1±0.5% *versus*

4±0.5% DMSO kontroll, $p < 0.02$, n=4). A bejuttatott idegen anyag, ez esetben a plazmid DNS apoptózist indukál, ami TUNEL assay-vel detektálható (*10.B ábra*). A különböző gátlószerek -beleértve a ATA univerzális endonukleáz gátlót- az apoptózist bizonyos mértékben csökkentették (ATA: 2.8±0.4%, Zn-Cl_2: 2.1±0.5%, Zn-NAC: 2.2±0.5% *versus* H_2O+pECFP-N1 kontroll: 4±0.4%; PFT: 3.9±1%, Z-VAD-fmk: 1.5±0.4%, *versus* DMSO+pECFP-N1: 6.9±1.9%, $p < 0.01$, n=4).

A kapott értékekből arra következtethetünk, hogy az apoptózis folyamatában részt vevő elemek speciális gátlásával a génbevitel esélyei növelhetők.



***10. ábra*. Az apoptózis-inhibitorok hatása a DNS transzfekcióra és a sejthalálra.** A TKPTS sejteket mindkét panelben 10nM ATA, 10µM $ZnCl_2$, 3µM Zn-NAC, 50µM PFT, 100µM Z-VAD-fmk hozzáadásával kezeltük.

V. MEGBESZÉLÉS

Kísérleteink arra irányultak, hogy a DNáz I és az EndoG aktivitásának csökkentésével megvédjük a gazdasejtbe bejuttatni kívánt "idegen" DNS-t. A génterápiák problémájának e látszólagosan egyértelmű megközelítése, vagyis az endonukleáz enzimek sejtvédő hatásának kikapcsolása nem merült fel korábban, egy beszámolótól eltekintve, amely egy másik endonukleázzal, a DNáz gammával kapcsolatos (Wilber és mtsai., 2002).

Korábbi adataink alapján a DNáz I-et tekintjük a legjelentősebb és az EndoG-t pedig a második legjelentősebb endonukleáznak vesesejtekben (Yin és mtsai., 2007). A plazmid, - és az idegen DNS bejuttatható a sejtekbe, de endonukleolitikus támadásnak vannak kitéve a sejten belül és kívül is. A Lipofektamin növeli a transzfekciók hatékonyságát, de nem védi a plazmid DNS-t az endonukleázok lebontásától. A sejtekben lévő endonukleáz aktivitás magasabbnak bizonyult a sejtek által kiválasztott, médiumban megjelenő endonukleáz aktivitásnál.

Megfigyeltük, hogy a plazmid incision assay által kalkulált teljes endonukleáz aktivitás szignifikánsan magasabb volt primer PTE sejtekben, mint immortalizált TKPTS sejtben. Ez magyarázatul szolgálhat primer sejtkultúrák transzfekció elleni rezisztenciájára, amit számos esetben leírtak korábban (Stacey és mtsai., 1993; Welter és mtsai., 2004; Zhong és mtsai., 2005). Ezek a megfigyelések korrelációba hozhatók korábbi adatainkkal, miszerint vesekivonatok endonukleáz aktivitása magasabb a primer sejtekénél (Basnakian és mtsai., 2005), ami ahhoz a konklúzióhoz vezet, hogy az endonukleáz aktivitás a következő sorrendben csökken:

Vese sejt > primer sejt> immortalizált sejt.

Gének bejuttatása a vesébe és veséből izolált primer sejtekbe sokáig megoldatlan problémát jelentett a magas DNáz szintnek köszönhetően. (Basnakian és mtsai., 2005; Djurovic és mtsai., 2004; Kalinowska és mtsai., 2005). Más primer sejtek transzfektálhatósága is ugyanilyen gondot jelentett, mivel a hatékonyság növelése speciális technikákat igényel a sejtek rezisztenciájának legyőzése érdekében. Ez a nehézség a primer makrofágoknál is fennáll (Stacey és mtsai., 1993), amelyek magas DNáz aktivitásukról ismeretesek. Rádiófrekvenciás elektroporáció nemrég hatékonynak bizonyult primer chondrocyták esetén (Welter és mtsai., 2004). Primer szarvasmarha endotél sejtek transzfektálhatók lineáris PEI-PEG-PEI triblock kopolimerek használatával (Zhong és mtsai., 2005).

A mi stratégiánk az idegen gének sejtekbe történő bejuttatására az internalizált plazmid DNS és siRNS túlélésén alapul. Ezt bizonyítja az az eredményünk, hogy a DNáz I KO és az EndoG KO sejteknek szignifikánsan magasabb a transzfekciós rátája a WT sejtekénél. Továbbá EndoG KO sejtekben a fluorescens siRNA transzfekció magasabb, mint a WT sejtekben. Ez arra utal, hogy az EndoG-nek kettős szerepe van a sejtvédekezésben: amellett, hogy lebontja az idegen DNS-t, megvédi a sejteket a DNS és RNS felvételtől.

Az extracelluláris DNáz I gátlása G-aktinnal nincs hatással a DNS transzfekció hatékonyságára jelezve, hogy az intracelluláris DNáz I az elsődleges molekuláris szereplője az idegen DNS-elleni gazdasejt védelemnek. Bár nem kérdéses, hogy az endonukleázok fontos/döntő szereppel bírnak az “idegen” és saját DNS (pl. apoptózis, nekrózis) lebontásában, mégis felmerül a kérdés, hogy egyéb sejtfaktorok részt

vesznek-e az örökítőanyagok degradációjában, befolyásolva a transzfektálhatóságot.

Kísérletesen alátámasztottuk, hogy a vesében a DNáz I szükséges az EndoG indukálásához (Yin és mtsai., 2007). Okkal gondolhatjuk, hogy ez a két endonukleáz együttműködhet a sejt védekezésében.

Az apoptózis a szervezet homeosztázisának fenntartására irányuló folyamat, számos fiziológiai esemény résztvevője. Külső jellel indukált formája sokféle lehet, de mind közül a legszélesebb körben ismert tulajdonsága a genomikus DNS lebomlása. A hasításért felelős enzimek azonosítása vita tárgyát képezi. Számos endonukleázt írtak le, mint a fragmentációért felelős enzimet, például a thymocyták Ca^{2+}-Mg^{2+}-dependens endonukleázait (Montague és mtsai., 1994; Pandey és mtsai., 1997; Peitsch és mtsai., 1993; Shiokawa és mtsai., 1994). a Mg^{2+}-dependens, Ca^{2+}-independens endonukleázok humán myeloid sejtvonalak apoptózisában vesznek részt (Kawabata és mtsai., 1997). Az endonukleázok lehetnek caspase-aktiváltak, vagy részt vehetnek caspase-independens apoptózisban (Padron-Barthe és mtsai., 2007). Annak eldöntése, hogy egy adott endonukleáz DNS bontása mely sejt apoptózisának melyik részét teszi ki, további kísérletek tárgyát képezi. TKPTS sejteken folyatott kísérletünkkel az apoptózis fontos résztvevőinek, a nukleázok aktivitását gátoltuk és ezzel a génbevitelt könnyítettük meg.

Összefoglalva eredményeink tükrözik a DNáz I és EndoG sejtvédekezésben betöltött szerepét a génbejuttatás szempontjából primer tubuláris epitél sejtek esetén. A jövőben szeretnénk tisztázni, hogy a két endonukleáznak milyen a kölcsönhatása és együttműködésének

természete. Az endonulkeázok ideiglenes és célzott gátlása új lehetőséget nyújthat a DNS stabilitás javítására génbevitel folyamán.

VI. KÖSZÖNETNYILVÁNÍTÁS

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VII. HIVATKOZÁSOK

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VIII. A JELÖLT TUDOMÁNYOS TEVÉKENYSÉGE

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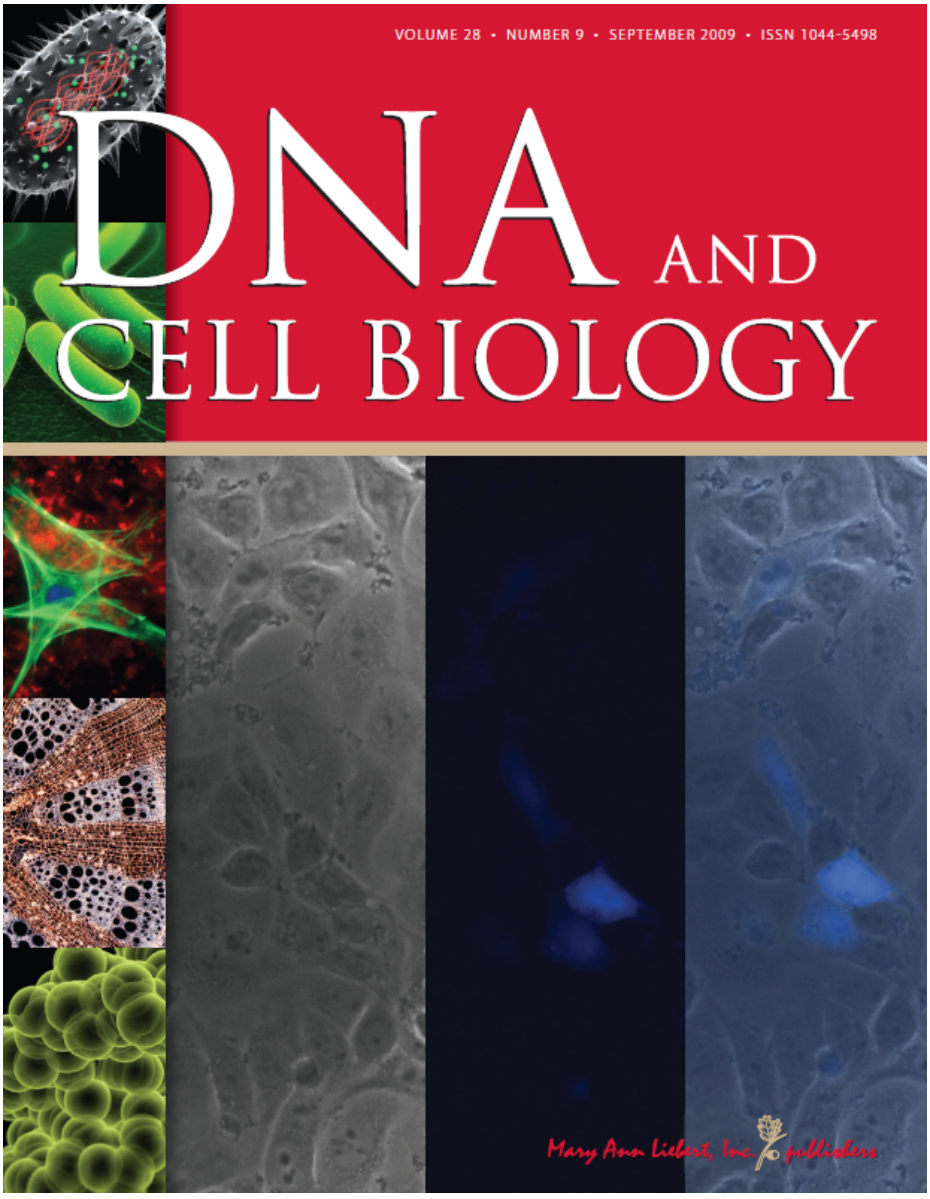
IX. SUMMARY

Gene delivery to kidney cells is essential for the development of gene therapy of nephropathy. Maintaining DNA stability is crucial for successful gene delivery. Endonucleases are known to play a role in degrading "foreign" DNA imported to the cell. However, what these DNases are and whether inactivation of these enzymes can improve gene delivery has not been examined. We have recently shown that two pro-apoptotic endonucleases, deoxyribonuclease I (DNase I) and endonuclease G (EndoG) are responsible for more than 90% of the total endonuclease activity in mouse kidney. Since primary cells are notoriously resistant to transfections, we studied DNA stability during gene delivery to primary renal tubular epithelial cells isolated from mice. We found that as compared to immortalized mouse tubular epithelial (TKPTS) cells, primary cells had higher total endonuclease activity. Endonuclease activity was present both in total cellular protein extracts (DNase I, EndoG and other DNases) and in culture media (mainly DNase I). Pre-treatment with Lipofectamine did not protect plasmid DNA against *in vitro* digestion by endonucleases. DNase I- or EndoG-deficient primary tubular epithelial cells isolated from knockout mice showed significantly higher rate of transfection by Lipofectamine-packed pECFP-N1 plasmid DNA than wild-type cells. We examined whether specific inhibition of these endonucleases may improve DNA stability during gene delivery. The transfection efficiency was increased by apoptosis inhibitors. Complete inhibition of extracellular (secreted) DNase I by G-actin did not improve plasmid transfection, which indicates that only intracellular DNase I is important for DNA stability. These data demonstrate the important role of the cytotoxic endonucleases in host cell defense against gene delivery in primary tubular epithelial cells.

X. FÜGGELÉKEK



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Uptake of Foreign Nucleic Acids in Kidney Tubular Epithelial Cells Deficient in Proapoptotic Endonucleases

Timea Buzder,[1,2] Xiaoyan Yin,[1] Xiaoying Wang,[1] Gaspar Banfalvi,[2] and Alexei G. Basnakian[1,3]

Degradation of DNA during gene delivery is an obstacle for gene transfer and for gene therapy. DNases play a major role in degrading foreign DNA. However, which of the DNases are involved and whether their inactivation can improve gene delivery have not been studied. We have recently identified deoxyribonuclease I (DNase I) and endonuclease G (EndoG) as the major degradative enzymes in the mouse kidney proximal tubule epithelial (TKPTS) cells. In this study, we used immortalized mouse TKPTS cells and primary tubular epithelial cells isolated from DNase I or EndoG knockout (KO) mice and examined the degradation of plasmid DNA during its uptake. DNase I and EndoG KO cells showed a higher rate of transfection by pECFP-N1 plasmid than wild-type cells. In addition, EndoG KO cells prevented the uptake of fluorescent-labeled RNA. Complete inhibition of secreted DNase I by G-actin did not improve plasmid transfection, indicating that only intracellular DNase I affects DNA stability. Data demonstrate the importance of DNase I and EndoG in host cell defense against gene and RNA delivery to renal tubular epithelial cells *in vitro*.

Introduction

Extracellular DNA uptake occurs during normal and cancer tissue growth (Bergsmedh *et al.*, 2006; Yan *et al.*, 2006) and during viral and bacterial infections (Chu *et al.*, 2006; Metifiot *et al.*, 2007), and is routinely used in genetic manipulations and experimental animals (Tanswell *et al.*, 1998; Glasspool-Malone *et al.*, 2002; Freitas *et al.*, 2007). The entry of foreign DNA (fDNA) is harmful to the host cell (Li *et al.*, 1999), causing DNA-dependent cell death, whereas DNase treatment before transfection prevented cell death (Stacey *et al.*, 1993). The introduction of single-stranded DNA in cells induced DNA damage and apoptotic factors, acting upstream of ATM/p53 in the p53-dependent pathway (Nur *et al.*, 2003). Studies have also demonstrated that the introduction of fDNA induces mutations due to homologous recombination (Thomas and Capecchi, 1986; Torchilin, 2006).

The uptake of DNA is restricted by a number of cell defense enzymes, the core of which consists of DNA endonucleases (Tanswell *et al.*, 1998; Glasspool-Malone *et al.*, 2002). Despite attempts to protect the fDNA by modifications, lipid or viral packaging, increased rate of DNA delivery, or by precise targeting to a tissue, our knowledge about the protection against the cellular defense system remained insufficient (Tanswell *et al.*, 1998; Glasspool-Malone *et al.*, 2002; Freitas *et al.*, 2007). Endocytosis of DNA normally leads to its lysosomal delivery, with DNases playing major role in degrading fDNA (Torchilin, 2006). However, it is still unknown which of these DNases are involved in the degradative process, and it was not decided whether the inactivation of these enzymes would improve gene delivery due to the lack of tools such as knockout (KO) endonuclease-deficient mice or endonuclease inhibitors.

In our previous studies we have selected those two of the nine known cellular cytotoxic endonucleases, which can degrade nonmodified DNA, namely, deoxyribonuclease I (DNase I) and endonuclease G (EndoG). These two were the most active endonucleases in kidney tubular epithelial cells (Peitsch *et al.*, 1995; Basnakian *et al.*, 2005; Irvine *et al.*, 2005). DNase I is a 31 kDa cytoplasmic enzyme that digests single- and double-stranded DNA. It can be excreted from the cells. Maximal enzymatic activity of DNase I can be measured when both Ca^{2+} and Mg^{2+} ions are present (Basnakian *et al.*, 2002). EndoG is a nuclear-encoded mitochondrial nuclease that translocates to the nucleus during apoptosis (Zhang *et al.*, 2003), is expressed in the cytoplasm as a precursor (33 kDa), and is converted to its mature form (28 kDa) upon entering the mitochondrion (Ikeda and Kawasaki, 2001). EndoG is a preferentially Mn-dependent enzyme (Widlak *et al.*, 2001) that is capable of digesting double- and single-stranded DNA, RNA, and DNA/RNA heteroduplexes (Huang *et al.*, 2006).

As primary cells are known to be resistant to DNA transfection, we have improved DNA delivery to primary

[1]Division of Nephrology, Department of Internal Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas.
[2]Department of Microbial Biotechnology and Cell Biology, University of Debrecen, Hungary.
[3]Central Arkansas Veterans Healthcare System, Little Rock, Arkansas.

tubular epithelial (PTE) cells using Lipofectamine. To determine the role of DNase I and EndoG, PTE cells were isolated from DNase I or EndoG KO mice. Our data provide evidence that in DNase I KO and EndoG KO cells the efficiency of transfection by plasmid DNA is significantly higher than in wild-type (WT) cells, indicating that these two endonucleases restrict DNA uptake in murine renal cells.

Materials and Methods

Animals

Homozygous DNase I KO mice (CD-1 background) were obtained from Dr. T. Moroy, University of Essen, Germany. EndoG KO mice (129xC57/B6 background) were obtained from Drs. M. Xu and J. Zhang, University of Cincinnati, OH. Because EndoG$^{-/-}$ animals are not viable, the cells were isolated from heterozygous mice (EndoG KO). DNase I KO mice were bred as heterozygotes, and EndoG$^{+/-}$ mice were bred with WT mice (EndoG$^{+/+}$). All mice were genotyped by PCR as previously described (Zhang *et al.*, 2003; Djurovic *et al.*, 2004). All animal experiments received human care to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences. All animal experiments were approved by the Animal Care and Use Committee of the Central Arkansas Veterans Healthcare System.

Cell cultures

Mouse PTE cells were freshly isolated from DNase I KO, EndoG KO, and WT mice as described by Nowak *et al.* (2003), and cultured up to 10 days (passages 1 and 2) before experiments. Immortalized mouse kidney proximal tubule epithelial (TKPTS) cells were obtained from Dr. Elsa Bello-Reuss (University of Texas Medical Branch, Galveston, TX) and were cultured as previously described (Ernest and Bello-Reuss, 1995). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/HAM F-12 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 7% fetal bovine serum (Hyclone, Logan, UT). Cells were maintained in a CO_2 incubator at 37°C in 5% CO_2, fed at 48–72 h intervals, and used within 1 day after reaching confluency, with the exception of RNA interference (RNAi) experiments described below.

Total protein extraction from cultured cells

Cells (2–4×10^6) were grown as described above. Cultured cells were collected by centrifugation (1000 rpm, 3 min, 4°C). After removal of supernatant, the cells were resuspended in phosphate buffered saline and centrifuged again as described above. For protein extraction, cells were diluted in 100 µL Buffer A (50 mM Tris-HCl, pH 7.9; 0.25 M sucrose) and the Complete Mini Proteinase Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (1 tablet/10 mL), and disrupted with Virsonic 475 (Virtis, Gardiner, NY) (2×20 s). Particulate material was precipitated out from the extract by centrifugation (14,000 rpm, 10 min, 4°C), and the supernatant was collected. The extracts were dialyzed against storage buffer (55% Glycerol, 10 mM Tris-HCl pH 7.6, 0.5 mM DTT) and stored at −20°C for up to 2 weeks without loss of endonuclease activity. Protein was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Bovine serum albumin was used as standard.

Plasmid incision assay

Endonuclease activity in total protein extracts from kidney cells and in culture medium was determined using the plasmid incision assay (PIA) with pBR322 plasmid (New England Biolabs, Beverly, MA) as substrate as described previously (Basnakian *et al.*, 2005).

To determine whether Lipofectamine is protecting plasmid DNA from degradation by endonucleases, pBR322 plasmid was pretreated with Lipofectamine before it was exposed to the culture medium. Plasmid and Lipofectamine were diluted separately in serum-free DMEM/HAM F-12 medium (Sigma-Aldrich), mixed together, and incubated for 20 min at room temperature.

After adding serially diluted samples (1:5) to the reaction mixture (1 µg pBR322 plasmid DNA, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 0.5 mM dithiothreitol) the reaction was incubated for 1 h at 37°C, after which the reaction was terminated by adding Stop-solution (10 mM Tris-HCl, pH 7.4, 1% SDS, 25 mM Na_2EDTA, 7.5 mM bromophenol blue). The samples were run on a 1% agarose gel in Tris-acetate-EDTA buffer, pH 8 (7V/cm, 35 min), and the DNA was viewed with ethidium bromide. The EagleEye scanning densitometer (Stratagene, La Jolla, CA) was utilized to quantify the relative amount of endonuclease-treated plasmid DNA present in a covalently closed circular DNA (C), open circular DNA (O), or linear DNA (L), or create a digested form (D). One unit was the amount of endonuclease capable of converting 1 µg covalently closed supercoiled plasmid DNA to open circular, linear, or digested DNA in 1 h at 37°C.

This assay was also used for the characterization of endonucleases in primary cells. Endonuclease activity was measured the same way as above in samples containing serially diluted protein (1:5), 1 µg plasmid pBR322 DNA (New England Biolabs), 2 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, and 0.5 mM dithiothreitol to determine the Ca/Mg-dependent (primarily DNase I) endonuclease activity or 5 mM $MnCl_2$, 10 mM Tris-HCl (pH 7.4), and 0.5 mM dithiothreitol for the Mn-dependent (mainly EndoG) activity.

As opposed to PIA, zymogram gel electrophoresis, previously used by us to assess DNase I activity (Basnakian *et al.*, 2005), was not applicable for EndoG due to low specific activity of the enzyme in the used tubular epithelial cells.

Real-time reverse transcriptase polymerase chain reaction

Our previously described protocol was followed (Basnakian *et al.*, 2006). Briefly, 1 µg of total RNA was reverse-transcribed in a 50-µL reaction mixture followed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) in a 25-µL reaction using SmartCycle (Cepheid, Sunnyvale, CA). Reaction mixture was prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Primers for endonucleases were as follows: 5′-GATGAGACCATCCCTC TGGA-3′ and 5′-ATGTGAGTC AGCCCATCTCC-3′ for EndoG, and 5′-ACTCAATCGGGACAAACCTG-3′ and 5′-ATT TCCACA GGGTTCACAGC-3′ for DNase I. Two-temperature cycles with annealing/extension temperature at 62°C for EndoG and DNase I, and 64°C for 18S mRNA were used. The fluorescence was measured at the end of the annealing step. The melting curve analyses were performed at the end

of the reaction after the 45th cycle between 60°C and 95°C to assess the quality of the final PCR products. The threshold cycle C(t) values were calculated by fixing the basal fluorescence at 15 units. cDNA samples for real-time PCR were diluted to 1:5, 1:10, and 1:200 for EndoG, DNase I, and 18S mRNA, respectively. Three replication reactions were performed for each sample, and the average C (t) was calculated. The standard curve of the reaction effectiveness was plotted against serially diluted (five points) mixtures of amplified cDNA samples for EndoG and for 18S mRNA. Calculation of the relative RNA concentration was performed using Cepheid SmartCycle software (Version 2.0d). Data are presented as ratio of EndoG/18s mRNA or DNase I/18s mRNA.

Plasmid transfection

PTE cells were transfected with pECFP-N1 plasmid (Clontech Laboratories, Mountain View, CA) that encodes cyan fluorescent protein (CFP) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were seeded into six-well plates 24 h before transfection. Briefly, 4 μg plasmid DNA and 1:7.5 DNA/liposome ratios were diluted in separate tubes in 250 μL serum-free DMEM/HAM F-12 medium (Sigma-Aldrich), mixed together, and incubated for 20 min at room temperature. Two milliliters serum-free DMEM/HAM F-12 medium (Sigma-Aldrich) was added to the cells. Transfection complexes were then added dropwise onto the cells. After 24–48 h incubation at 37°C in 5% CO_2 the expression of CFP was detected by fluorescent microscopy using cyan filter.

Small interfering RNA transfection

PTE cells were transfected using TransIT-TKO transfection reagent (Mirus Bio, Madison, WI). Cells were seeded into six-well plates 24 h before transfection. In brief, 18 μL transfection reagent was diluted to 250 μL with serum-free DMEM/HAM F-12 medium (Sigma-Aldrich) incubated for 15 min, and then 75 μL (1 μM) small interfering RNA (siRNA)/fluorescent siRNA (Label IT RNAi Delivery Control-Fluorescein; Mirus Bio) was added and incubated for further 20 min at room temperature. After incubation 1172 μL serum-free DMEM/HAM F-12 medium (Sigma-Aldrich) was added to the cells. The transfection complex was then added dropwise to the cells. After 48 h incubation at 37°C in 5% CO_2 the expression of fluorescent-labeled siRNA was detected by fluorescent microscopy.

The Label IT RNAi Delivery Control–Fluorescein contains a chemical dye fluorescein, also known as Fluorescein Isothiocyanate. The Fluorescein Isothiocyanate label is attached via a linker molecule and covalently bonded to the nucleotides. The introduction of short RNA duplexes into mammalian cells leads to sequence-specific destruction of target mRNA. These short double-stranded RNAs, referred to as siRNA, which can act catalytically at sub-molar ratios to cleave greater than 95% of the target mRNA in the cell and destruction of the mRNA target, can ultimately lead to decreased expression of the encoded protein. The RNAi effect can be long-lasting and may be detectable after many cell divisions. These properties make siRNA extremely effective at inhibiting target gene expression once introduced into the cell. The sequence of the Label IT RNAi Delivery Control is not homologous to any known mammalian gene and is not known to affect any cellular events. It is designed as a tool to facilitate observation and optimization of double-stranded RNA oligonucleotide delivery during RNAi experiments, both *in vitro* and *in vivo* (see manufacturer protocol; Mirus Bio, Lit.# ML039).

TKPTS cells were transfected using TransIT-TKO transfection reagent (Mirus Bio). Cells were seeded into 24-well plates 24 h before transfection. In brief, 4 μL transfection reagent was diluted to 50 μL with serum-free DMEM/HAM F-12 medium (Sigma-Aldrich) incubated for 15 min, and then 15 μL (1 μM) siRNA was added and incubated for further 20 min at room temperature. After incubation 250 μL serum-free DMEM/HAM F-12 medium (Sigma-Aldrich) was given to the cells. The transfection complex was then added dropwise to the cells. After 48 h incubation at 37°C in 5% CO_2 (medium was replaced-medium with serum-after 2 h) cells were transfected with enhanced CFP plasmid, pECFP-N1 (see Plasmid transfection section in Materials and Methods). After 24–48 h incubation at 37°C in 5% CO_2 the expression of CFP was detected by fluorescent microscopy using cyan filter.

EndoG siRNA target sequence was AAAUGCCUGGAA CAACCUUGA, DNase I siRNA target sequence was TGA CATCGCTGTTATCCAA (Dharmacon, Lafayette, CO), and siCONTROL was Non-Targeting siRNA (Dharmacon).

Statistical analysis

Statistical analysis was performed with a two-way ANOVA and Student's *t*-test. Results were expressed as mean ± standard error of the mean. $p < 0.05$ was considered significant.

Results

Endonuclease activity of TKPTS cells to digest plasmid DNA

Initial experiments served to determine whether plasmid DNA can be digested by endonucleases present in tubular epithelial cells and excreted to the culture medium. After growing TKPTS cells to confluency, cells and medium were collected separately. Total cellular protein was extracted as described in the Materials and Methods section. Protein concentrations were measured in medium and protein extracts. Endonuclease activity was measured using the PIA. In this experiment, the circular covalently closed and supercoiled pBR322 plasmid DNA was converted by single-stranded breaks and double-stranded breaks to open circular (O) and linear (L) forms, respectively (Fig. 1A). The activity was higher in cell protein extracts than in the external medium, indicating that the destruction of plasmid DNA takes place mainly inside the cells.

Lack of inhibition of extracellular endonuclease activity by lipofectamine

Lipofectamine is preferentially used for plasmid DNA delivery during transfection of cells (Djurovic *et al.*, 2004). To test whether Lipofectamine is capable of protecting plasmid DNA from degradation by endonucleases, pBR322 plasmid was pretreated with Lipofectamine and then exposed to the culture medium. As shown in Figure 1A (right panel), Lipofectamine *per se* provided no protection against the endonucleases present in culture medium.

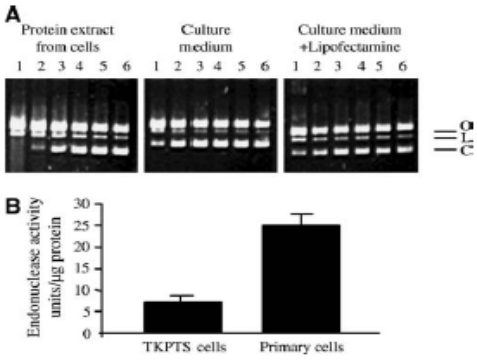

FIG. 1. Endonuclease activity in tubular epithelial cell extract and culture medium. The activity was measured using pBR322 PIA as described in the Materials and Methods section. (**A**) Endonuclease activity is present both in the cellular protein extracts and in the culture medium (left and middle panels). Pretreatment with Lipofectamine does not protect plasmid DNA against *in vitro* digestion by endonucleases (right panel). Dilutions (1–6) of cell extract or medium 1:1, 1:5, 1:25, 1:125, 1:625, and 1:3125, respectively. O, open circular DNA (with one or more single-strand DNA breaks but no double-strand breaks); L, linear DNA (with one double-strand DNA break); C, covalently closed circular DNA (without DNA breaks), which is the primary substrate for endonucleases. Endonuclease activity is seen only in the first two dilutions in cell extract, and in nondiluted culture medium. (**B**) Endonuclease activity in immortalized TKPTS cells and PTE cells. Primary cells have a higher total endonuclease activity (25 ± 2 units/μg protein in primary cells vs. 7 ± 3 units/μg protein in TKPTS cells, $n = 3$–6, $p < 0.001$) as measured using the pBR322 PIA in the presence of Ca^{2+} and Mg^{2+} ions (2 mM $CaCl_2$ and 5 mM $MgCl_2$), which are the cofactors for most of the cellular endonucleases. PIA, plasmid incision assay; TKPTS, mouse kidney proximal tubule epithelial; PTE, primary tubular epithelial.

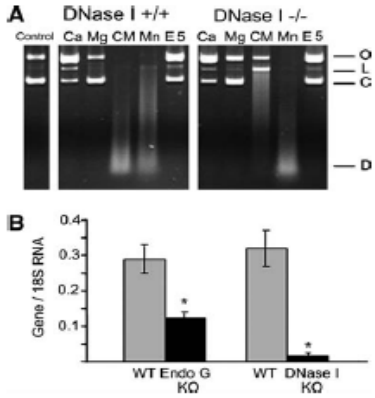

FIG. 2. Activity and expression of endonucleases in PTE cells. (**A**) In the total protein extracts isolated from DNase I WT mice, the strongest endonuclease activity could be obtained when Ca^{2+} and Mg^{2+} ions were added together, resulting in digested DNA. This is characteristic to DNase I, which therefore provides most of the endonuclease activity in the normal kidney. In the kidney tissue extracts obtained from DNase I KO mice, Mn-dependent endonuclease was the most prominent, suggesting that EndoG is the second major endonuclease in the absence of DNase I (Widlak *et al.*, 2001). Vertical row: O, open circular DNA; L, linear DNA; C, covalently closed circular DNA; D, digested DNA. Horizontal row: control nondigested pBR322 DNA; Ca^{2+}, 2 mM $CaCl_2$, pH 7.5; Mg^{2+}, 2 mM $MgCl_2$, pH 7.5; CM [Ca^{2+}+Mg^{2+}], 2 mM $CaCl_2$ + 2 mM $MgCl_2$; Mn^{2+}, 2 mM $MnCl_2$, pH 7.5; E5, 2 mM EDTA, no cations, pH 5 (to measure DNase II activity). (**B**) Expression of endonucleases in WT, EndoG KO, and DNase I KO cells measured using real-time reverse transcriptase polymerase chain reaction. DNase I expression is wiped out almost completely, while EndoG KO is partially inhibited because these cells were isolated from heterozygous animals ($n = 4$, $^*p < 0.001$). DNase I, deoxyribonuclease I; WT, wild-type; KO, knockout; EndoG, endonuclease G.

Endonuclease activity in immortalized versus primary cells

Primary cells are known to exert some resistance to DNA transfection (Stacey *et al.*, 1993; Welter *et al.*, 2004; Zhong *et al.*, 2005). To determine whether the resistance to fDNA is associated with the high endonuclease activity in primary cells, we compared the total endonuclease activities of protein extracts isolated from immortalized TKPTS cells and from PTE cells.

The PIA shows that endonuclease activity was several times higher in primary cells than in immortalized cells (Fig. 1B), confirming our notion that the resistance of primary cells to transformation is primarily due to their nuclease activity. This conclusion is based on the assumption that the permeability of plasmid to membrane is the same in primary and TPKTS cells.

Endonucleases in KO primary cells

As endonucleases have overlapping cation and pH requirements, direct comparison of the activities characteristic to specific endonucleases is usually impossible. Therefore, the use of KO mice provides a unique opportunity to determine individual endonuclease activities belonging to particular endonucleases. DNase I and EndoG endonucleases were chosen since they turned out to be the two most active ones in murine kidney cells (Basnakian *et al.*, 2005; Yin *et al.*, 2007). PTE cells were isolated from WT and KO mice as described, and their protein extracts were tested for endonuclease activities using the PIA in the presence of different cations. Results show that the majority of the endonuclease activity in WT mice is Ca/Mg-dependent DNase I, the major endonuclease in these cells (Fig. 2A). After inactivation of DNase I in DNase I KO cells, the second most active endonuclease could be detected as Mn-dependent endonuclease, corresponding to EndoG. Partial inactivation of EndoG in heterozygous EndoG KO cells was associated with a reduced Mn-dependent activity without any effect on the Ca/Mg-dependent DNase I activity. However, the reduction of Mn-

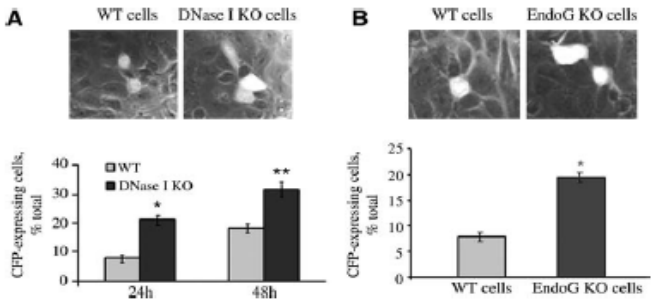

FIG. 3. Efficiency of plasmid transfection of PTE cells with active or inactive endonucleases. (**A**) Expression of CFP after pECFP-N1 plasmid transfection in DNase I KO PTE cells is higher than in WT cells. KO versus WT ($21 \pm 5\%$ vs. $8 \pm 5\%$ transfected cells, $n = 3$, $^{*}p < 0.013$; $32 \pm 6\%$ vs. $18 \pm 5\%$ transfected cells, $n = 3$, $^{**}p < 0.025$). Two time-points have been used, 24 h and 48 h incubation after transfection. The second time-point shows higher transfection efficiency and has been chosen for experiment B, where EndoG WT and KO cells have been transfected with the same plasmid. (**B**) Expression of CFP 48 h after pECFP plasmid transfection in EndoG KO PTE cells is higher than in WT cells ($19 \pm 5\%$ vs. $8 \pm 5\%$ CFP-positive cells, $n = 6$, $^{*}p < 0.001$). CFP, cyan fluorescent protein.

dependent activity in EndoG KO mice did not reach statistical significance, probably because the precision of PIA was not enough to measure incomplete inactivation of the enzyme in heterozygotes (data not shown). Real-time RT-PCR was performed as an alternative approach to determine whether the expression of these two endonucleases is decreased in murine KO cells. Our data indicate a complete fall out (95–100%) of DNase I activity in homozygous DNase I KO mice and a 60–70% loss in heterozygous EndoG in KO mouse kidney cells (Fig. 2B).

Inactivated DNase I and reduced EndoG contribute to DNA transfection

The inactivation of DNase I and/or reduced activity of EndoG may affect the transfection of DNA into PTE cells. To test this possibility we compared transfection efficiencies of WT and KO cells by introducing into these cells the pECFP plasmid that encodes the CFP. Figure 3A shows that more CFP entered in DNase I KO cells, indicating that the rate of transfection is significantly higher relative to WT cells. The transfection of EndoG KO cells was also significantly more efficient than that of WT cells (Fig. 3B). These data confirm that the presence of endonucleases reduces the efficiency of DNA transfection.

siRNA transfection

KO mice and cells are very valuable models, but the results obtained with them are often difficult to interpret, as the long-term effect of an inactive protein may influence the expression of other proteins. To confirm that the inactivation of DNase I or EndoG provides a beneficial effect on plasmid transfection, we have used siRNS to silence these endonucleases. These experiments proved that silencing of either endonuclease strongly increases the transfection efficiency of tubular epithelial cells.

TKPTS cells have been treated with DNase I siRNA, EndoG siRNA, or both and then transfected with pECFP-N1 (cyan) plasmid. The EndoG siRNA target sequence consisted of AAAUGCCUGGAACAACCUUGA. The DNase I siRNA target sequence corresponded to GACATCGCTGTTATCCAA (Dharmacon). Cells treated with DNase I siRNA, with EndoG siRNA or with DNase I + EndoG siRNA, exhibited significantly ($^{*}p = 0.001$) higher efficiency of transfection ($\approx 9\%$) with cyan plasmid, than the siRNA control ($\approx 2.5\%$) or the transfection reagent (TKO) control ($\approx 4\%$) (Fig. 4). These experiments proved that the silencing of either endonuclease strongly increases transfection efficiency of TKPTS cells.

Inactivation of EndoG promotes RNA transfection

As opposed to DNase I, EndoG degrades both DNA and RNA (Kalinowska *et al.*, 2005). Consequently, besides its anti-DNA activity, EndoG may also be an important factor in RNA transfection of cells. To test the validity of this assumption EndoG KO and WT cells were transfected with a fluorescein-labeled siRNA (Label IT RNAi Delivery Control shRNA; Mirus Bio). The Label IT RNAi Delivery Control consists of

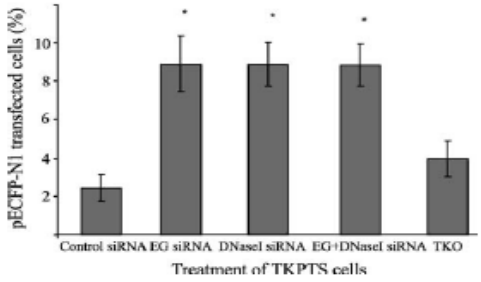

FIG. 4. Efficiency of pECFP-N1 plasmid transfection in TKPTS cells after EG siRNA, DNase I siRNA, and EG+DNase I siRNA treatment. TKPTS cells were treated with EndoG, DNase I, and EndoG+DNase I siRNA using TransIT-TKO transfection reagent (TKO), controlled with siCONTROL Non-Targeting siRNA (control siRNA) and transfected with pECFP-N1 cyan plasmid. SiRNA-silenced cells (EG, DNase I and both) show significantly higher plasmid transfection efficiency (8.8% vs. 2.4% of pECFP-N1 transfected cells, $^{*}p = 0.001$). siRNA, small interfering RNA.

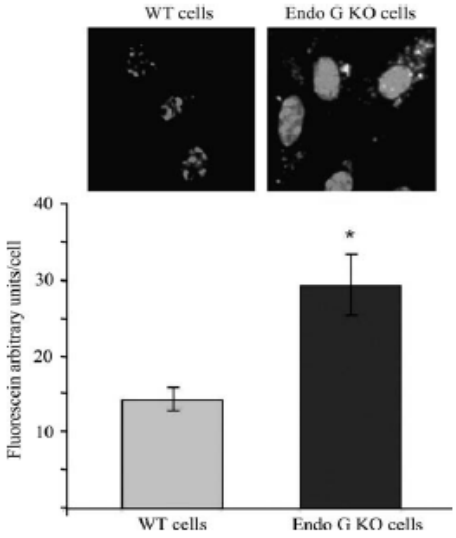

FIG. 5. Efficiency of RNA transfection of PTE cells with active or inactive EndoG. Primary EndoG KO or WT mouse tubular epithelial cells were transfected with fluorescent siRNA as described in the Materials and Methods section. About 48 h later RNA transfection was detected using a fluorescent microscope. Blue color of DAPI was used to stain the nuclei. EndoG KO cells show significantly higher rate of siRNA transfection than WT cells (14 ± 2 vs. 29 ± 4 arbitrary fluorescence units per cell, $n = 3$, $^*p < 0.01$).

fluorescein-labeled double-stranded RNA duplexes that have the same length, charge, and configuration as standard siRNA used in RNAi studies. As suggested by the manufacturer, the sequence of this probe was not homologous to any known mammalian gene and was not known to affect any cellular events. In our experiment, EndoG KO cells had a two times faster rate of siRNA transfection than those of WT cells (Fig. 5). This observation suggests that EndoG is likely to play a dual role in host cell defense; it protects cells from both DNA and RNA invasion.

Inactivation of extracellular DNase I has no effect on transfection

While EndoG is entirely an intracellular enzyme, DNase I is secreted (Lacks, 1981). To determine whether secreted extracellular DNase I influences the efficiency of transfection, it was inhibited by G-actin, a known specific and irreversible inhibitor of DNase I (Lacks, 1981). When choosing G-actin, it was also taken into consideration that G-actin is not toxic to cells and it does not enter cells and thus can be applied specifically to interact with and eliminate the activity of extracellular DNase I. TKPTS cells were exposed to G-actin in the concentration range between 0 and 2 mg/mL in the medium. Endonuclease activity was measured after G-actin treatment using the PIA (Fig. 6A). Complete inhibition of DNase I activity was found at 1 mg/mL G-actin concentration. In another experiment TKPTS cells were treated either with 1 mg/mL G-actin or albumin as a control and then transfected with pECFP plasmid (Fig. 6B). There was no difference between G-actin and the control albumin treatment, suggesting that extracellular DNase I is unlikely to influence transfection and only intracellular DNase I seems to be involved in the anti-DNA host cell defense. Control cells were not treated, and albumin was used as a protein control. In the experiment there was no difference between the albumin control and G-actin treatment. This explains why extracellular DNase I inactivated by G-actin does not improve transfection.

Discussion

We have attempted to protect the fDNA to be introduced in host cells by reducing the DNase I and EndoG activities. This seemingly obvious approach has not been used earlier due to the lack of specific inhibitors and to insufficient data, regarding the potential of these enzymes as host cell defense enzymes. There is only one report showing the role of another endonuclease, DNase gamma (DNase 1L3) in host cell defense (Wilber *et al.*, 2002). Our earlier data showed that DNase I is the major endonuclease in tubular epithelial cells and EndoG is the second major degradative enzyme (Yin *et al.*, 2007). Plasmid as fDNA was introduced in host cells, but is subject to endonucleolytic attacks both outside and inside the cells. Lipofectamine increased the efficiency of

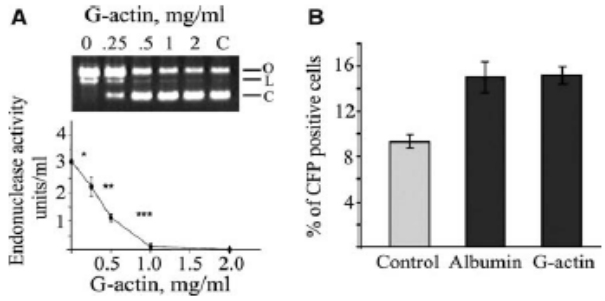

FIG. 6. Extracellular DNase I does not influence DNA transfection efficiency. (**A**) TKPTS cells were treated with different concentration of G-actin in culture medium, and endonuclease activity was measured with PIA. G-actin inhibited DNase I in culture medium ($n = 4$ per concentration point, $^*p = 0.018$, $^{**}p = 0.0052$, $^{***}p = 0.006$). (**B**) The efficiency of TKPTS cells transfection with pECFP plasmid in the presence of 1 mg/mL G-actin was not different from the one measured in the presence of 1 mg/mL albumin (15 ± 5 vs. $15 \pm 8\%$ of CFP-positive cells, $n = 6$).

transfection, but did not protect plasmid DNA from destruction by endonucleases. Cellular extracts turned out to have a much higher endonuclease activity than the nuclease excreted by the cells to the culture medium.

It is to be mentioned that in our experiments, fDNA was attacked by endonucleases, which are compartmentalized and thus are not freely available in the cytoplasm: EndoG is mainly localized in mitochondria, while DNase I is associated with endoplasmic reticulum. Therefore, either there are enough preexisting free cytoplasmic EndoG and DNase I activities to destroy the incoming DNA, or fDNA induces the release of these endonucleases from cellular compartments. Such a release could be part of the host cell apoptosis induced by fDNA. That the introduction of fDNA causes apoptosis has been described by Shimokawa *et al.* (2000).

We have also observed that the total specific endonuclease activity measured by the PIA was significantly higher in primary cells than in immortalized TKPTS cells. This could explain why primary cells are more resistant to transfection than immortalized cells. The resistance of primary cells to transfection was also reported by others (Stacey *et al.*, 1993; Welter *et al.*, 2004; Zhong *et al.*, 2005). Our strategy to introduce foreign genes in cells is based on the inactivation of the two major endonucleases to contribute to the survival of the internalized plasmid DNA and siRNA. That this approach has a perspective is proved by the observations that both DNase I KO and EndoG KO cells had significantly higher rate of transfection compared to WT cells. Moreover, in EndoG KO cells the rate of fluorescent siRNA transfection was also significantly higher than in WT cells. This suggests that EndoG has a dual role in host cell defense: besides degrading fDNA, it protects cells from both DNA and RNA uptake. Inhibition of extracellular DNase I by G-actin did not affect the efficiency of DNA transfection, indicating that only intracellular DNase I is the primary molecular player in the anti-DNA host cell defense. Although, there is no doubt that endonucleases play an important if not decisive role in the degradation of foreign and self-DNA (e.g., apoptosis and necrosis), the question arises whether cellular factors other than endo- and exonucleases are involved in the degradation of nucleic acids, their knocking out of which could contribute to the efficiency of transfection. In a recent study we have confirmed that in the kidney, DNase I is necessary for EndoG induction (Yin *et al.*, 2007). It is thus reasonable to think that there might be some cooperation between these two endonucleases during the protection of cells against the invasion of nucleic acids. The experiments carried out in murine renal system (WT and KO) determine future studies, which will be directed to (a) selection of specific drugs that inhibit DNase I and EndoG as a new strategy for efficient gene therapy in kidney cells, (b) assessment of the function of these endonucleases in apoptosis in the degradation of fDNA, and (c) to determine whether other factors could modulate DNase I or EndoG activities to increase transfection/transduction efficiency through the partial downregulation of endonucleases, and (d) another viable approach could be the suppression of fDNA-induced apoptosis and thus indirectly suppress other anti-fDNA endonuclease activities that may contribute to the DNA destruction. Although fDNA induced apoptosis and DNases in other studies (Stacey *et al.*, 1993; Nur *et al.*, 2003), the inhibition of apoptosis has not been previously used for improving the delivery of fDNA.

The delivery of fDNA into DNase I–deficient cells raises the question what the physiological consequences of impaired clearance of DNase I could be. According to an earlier view DNase I may protect against development of systemic *lupus erythematosus* (SLE), suggesting that DNase I treatment may be helpful in preventing the onset of the disease. Direct evidence was provided that deficient DNase I function may lead to *lupus erythematosus* (Napirei *et al.*, 2000). In addition, KO mice lacking DNase I develop antichromatin autoantibodies and glomerulonephritis. These observations indicate that DNase I may protect against SLE by digesting extracellular DNA (Napirei *et al.*, 2006). Although DNase I deficiency was shown to produce a *lupus*-like syndrome in mice and *lupus* patients often have low levels of DNase I, there is no evidence that any human SLE is caused by DNase I deficiency alone (Walport, 2000). Consequently, supplementing the enzyme is unlikely to offer SLE patients more than partial symptom relief, as DNase I deficiency is only one of the factors in *lupus* disorder (Napirei *et al.*, 2000).

In conclusion, our data reflect the importance of DNase I and EndoG in host cell defense against gene delivery in PTE cells. Future studies will shed light on the cooperative nature of these two endonucleases in destroying fDNA. Temporary and targeted inhibition of these endonucleases may provide new modalities to improve DNA stability during gene delivery.

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Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Gaspar Banfalvi
Department of Microbial Biotechnology and Cell Biology
University of Debrecen
1 Egyetem Square
Debrecen 4010
Hungary

E-mail: bgaspar@delfin.klte.hu

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Induction of Renal Endonuclease G by Cisplatin Is Reduced in DNase I-Deficient Mice

Xiaoyan Yin,* Eugene O. Apostolov,* Sudhir V. Shah,*† Xiaoying Wang,* Konstantin V. Bogdanov,* Timea Buzder,* Anna G. Stewart,* and Alexei G. Basnakian*†

*Division of Nephrology, Department of Internal Medicine, University of Arkansas for Medical Sciences, and †Central Arkansas Veterans Healthcare System, Little Rock, Arkansas

ABSTRACT

Nephrotoxicity from the chemotherapeutic drug cisplatin is associated with DNA fragmentation and cell death. We have recently demonstrated that DNase I knockout mice are significantly protected against cisplatin nephrotoxicity, but it is unknown whether the DNA fragmentation that occurs is produced by DNase I or another endonuclease. In this study we assessed the expression of several endonucleases involved in cell death after injection of cisplatin and found that the expression of endonuclease G (EndoG) increased whereas the expression of DNase I decreased almost to zero. Immunostaining showed that some nuclei contained both fragmented DNA and EndoG, suggesting that EndoG may cause DNA fragmentation induced by cisplatin. The increase in expression of EndoG was greater in wild-type mice than in DNase I knockout mice, indicating a potential link between the two endonucleases. In support of such a link, overexpression of DNase I in cultured mouse tubular epithelial cells also induced EndoG. Furthermore, gene silencing of EndoG *in vitro* provided significant protection against cell death. Taken together, our data suggest that both DNase I and EndoG mediate cisplatin injury to tubular epithelial cells.

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The nephrotoxicity of cisplatin (cis-diamminedichloroplatinum II) is the major factor limiting the use of this drug in the chemotherapy of cancer.[1–3] It is known that cisplatin accumulates in the kidney more that in other organs, and its toxicity to the kidney is dosage dependent.[4] The mechanism of cisplatin nephrotoxicity is not completely understood. It is known to be associated with DNA fragmentation determined either by a 200-bp DNA ladder in agarose[5,6] or by using the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay.[7–9] These types of DNA fragmentation are commonly attributed to apoptosis mediated by an unknown cell death endonuclease that is capable of internucleosomal DNA fragmentation and generation of 3′OH ends available for the TdT reaction.[10] Cell death endonucleases are a recently recognized group of enzymes that includes deoxyribonuclease I (DNase I), DNase γ, caspase-activated DNase (CAD), endonuclease G (EndoG), and DNase II.[11–15] They act both premortem, leading to cell death, and postmortem, providing a "clean-up" after cell death.[10] Because these two processes cannot be easily distinguished and there are no specific inhibitors for the endonucleases, the use of knockout (KO) models is the only available tool to study the role of premortem endonuclease-mediated events *in vivo*.

We previously showed that DNase I is the most

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Correspondence: Dr. Alexei G. Basnakian, University of Arkansas for Medical Sciences, Department of Internal Medicine, Division of Nephrology, 4301 W. Markham Street, #501, Little Rock, AR 72205. Phone: 501-257-5052; Fax: 501-257-4822; E-mail: basnakianalexeig@uams.edu

active cell death endonuclease among cell death endonucleases in the kidney.[16,17] Renal injury by ischemia-reperfusion was associated with the induction/activation of DNase I.[18,19] The suppression of DNase I by antisense was protective against hypoxia/reoxygenation injury to renal tubular epithelial cells *in vitro*.[18] Our recent study of cisplatin-induced renal injury demonstrated that DNase I KO mice are protected against cisplatin.[17] It remains unclear whether DNase I is the sole enzyme that produces DNA fragmentation and induces cell death or these processes require the participation of another endonuclease.

In this study, we demonstrated that DNA fragmentation induced by cisplatin strongly depends on the contribution of EndoG. EndoG is a nuclear-encoded mitochondrial enzyme that is known to be released from its original location, translocate to nucleus, and degrade nuclear DNA during caspase-independent apoptosis.[20,21] Importantly, we showed that the presence of DNase I before cisplatin injury was necessary for the induction of EndoG, which therefore serves as the executioner of DNase I–mediated cell death in the kidneys of mice that are treated by cisplatin. The expression of DNase I-cyan fluorescence protein (DNase I–CFP) fusion protein in immortalized mouse proximal tubule (TKPTS) cells caused the induction of EndoG, which provided additional evidence for the role of DNase I in EndoG regulation. In support of the role of EndoG as a downstream executioner of DNA fragmentation and kidney cell death, the EndoG activation was associated with increased DNA fragmentation *in vivo*, and the silencing of EndoG by anti-EndoG short interference RNA (siRNA) or peroxisome proliferator–activated receptor α (PPAR-α) agonist provided protection against the cisplatin injury of TKPTS cells *in vitro*.

RESULTS

Renal Endonucleases before Injury

This study began with the characterization of endonuclease activity in the kidneys. As determined by using the DNase I-specific single radial enzyme diffusion assay, inactivation of the DNase I gene (in DNase I KO mice) caused complete removal of DNase I activity from total kidney extracts (Figure 1A). Semiquantitative reverse transcriptase–PCR (RT-PCR) showed that DNase I is not expressed in KO mice, whereas other endonucleases are expressed at the same levels as in wild-type (WT) mice (Figure 1B). We found that in addition to DNase I, three other proapoptotic endonucleases—DNase II, CAD, and EndoG—are expressed in the kidney, whereas mRNA for DNase γ could not be detected.

In total protein extracts from nontreated WT mice, the strongest endonuclease activity was observed in the presence of Ca^{2+} and Mg^{2+} ions added together (Figure 1C). This is characteristic of DNase I, which is known to be the most active Ca/Mg-dependent endonuclease in mammalian tissues.[17] Therefore, DNase I provides most of the endonuclease activity

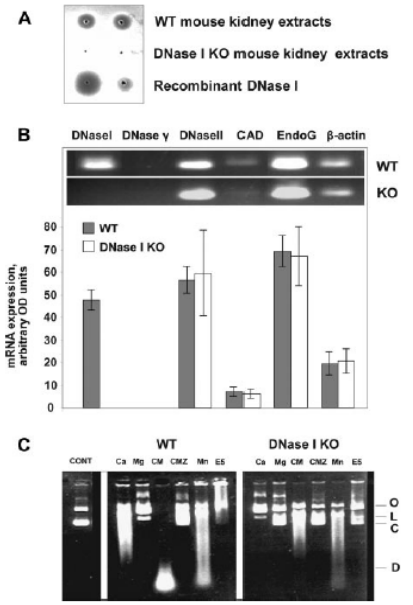

Figure 1. Expression of DNase I and other endonucleases in wild-type (WT) and DNase I knockout (KO) mice. (A) Single radial enzyme diffusion assay showed that protein extracts from WT kidneys contain active DNase I, whereas it is absent in KO kidney extracts. Human recombinant DNase I (Dornase; Genentech, South San Francisco, CA) was used as positive control. (B) Semiquantitative reverse transcriptase–PCR (RT-PCR) of cell death endonucleases expressed in normal kidneys of WT and DNase I KO mice. Products of the RT-PCR reaction were produced and separated in agarose gel as described in the Concise Methods (top) and then quantified by densitometry (bottom; $n = 4$ to 6 per group). (C) Inactivation of CaMg-dependent endonuclease DNase I activity (CM) as measured using plasmid incision assay in total protein extracts of DNase I−/− mice. Mn-dependent endonuclease G (EndoG) activity (Mn) is the most prominent in these mice. DNase II activity measured in EDTA at pH 5 (E5) is very low. O, open circular DNA; L, linear DNA; c, covalently closed circular DNA; D, digested DNA.

in the normal kidney. In kidney tissue extracts from KO mice, Mn-dependent endonuclease was the most prominent (Figure 1C, right), suggesting that EndoG, the only known Mn-dependent endonuclease, is the second major endonuclease in the absence of DNase I.

DNase I Is Suppressed during Cisplatin Injury In Vivo

For definition of the changes of renal endonucleases associated with cisplatin nephrotoxicity, renal failure was induced by a

single cisplatin (20 mg/kg) injection as described in our previous study.[17] Because cisplatin kidney injury has been reported in association with increased DNA fragmentation,[5,7,17] it was expected that the endonuclease activity would be increased after cisplatin injection. Contrary to our expectations, the endonuclease activity of total kidney extracts measured using plasmid incision assay in the presence of Ca^{2+} and Mg^{2+} ions was decreased at day 1 and then had a tendency to recover on later days (Figure 2A). Because Ca/Mg-dependent activity was previously shown to be mainly represented by DNase I,[17] these results suggested that DNase I activity is decreased after cisplatin injection. Mn-dependent endonuclease activity, which is known to belong to both DNase I[22] and EndoG,[15] remained unchanged after cisplatin treatment (Figure 2B). This suggested that while DNase I is downregulated, EndoG activity is increased during cisplatin injury. These data also showed that the increase of EndoG activity occurs only in DNase I–positive WT mice, whereas DNase I KO mice have very little induction of EndoG.

Urine DNase I is an important marker of DNase activity in the kidney because it is secreted by tubular epithelium.[23] The measurement of total CaMg-dependent endonuclease activity in urine showed that DNase I KO mice have only approximately 1% of endonuclease activity left, suggesting that urine DNase is represented almost entirely by DNase I (Figure 2C). The activity of DNase I in urine was decreased almost to zero within 4 h after cisplatin injection and remained suppressed up to 8 d after the impact, which explains the decrease of the total endonuclease activity. Urine DNase I also disappeared as determined by zymogram gel (Figure 2D).

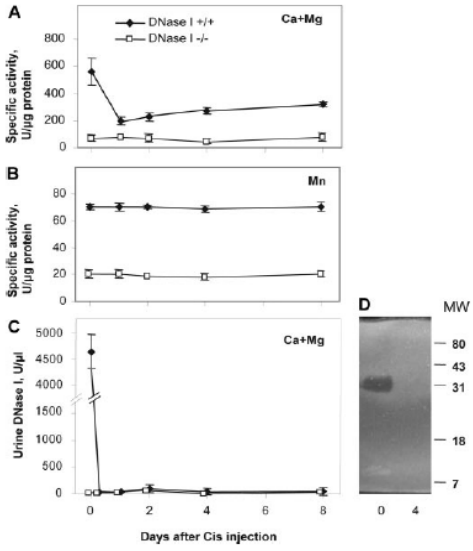

Figure 2. Endonuclease activity in total kidney protein extracts from DNase I KO and WT mice that were treated with a single dose of cisplatin (20 mg/kg). Activity was measured using plasmid incision assay in the presence of Ca^{2+} and Mg^{2+} (A) or Mn^{2+} ions (B) as described in the Concise Methods ($n = 6$ in each point). (C) Activity in urine measured using plasmid incision assay in the presence of Ca^{2+} and Mg^{2+} ($n = 6$ in each point). (D) Zymogram gel of kidney protein extract isolated at days 0 and 4 after cisplatin injection.

EndoG Is Induced during Cisplatin Injury in Mice Expressing DNase I

As determined by Western blotting, the expression of DNase II and CAD was not changed after cisplatin injection (Figure 3A). In contrast, EndoG was strongly induced on day 1 (data not shown) and reached a maximum 4 d later after cisplatin injection. As described next, EndoG induction correlated with DNA fragmentation and tubular necrosis as determined by histology. Importantly, EndoG was induced more in DNase I WT mice than in KO mice, suggesting that DNase I may be necessary for the induction of EndoG (Figure 3B).

Overexpression of DNase I In Vitro Causes Induction of EndoG

For studying whether DNase I can cause EndoG induction, cultured TKPTS cells were transfected with pECRF-DNaseI construct or empty pECFP vector as described previously. An alternative approach using silencing of DNase I seemed impossible considering the large number of DNase I–like proteins in cells.[11,24] The expression of the fusion protein of CFP was de-

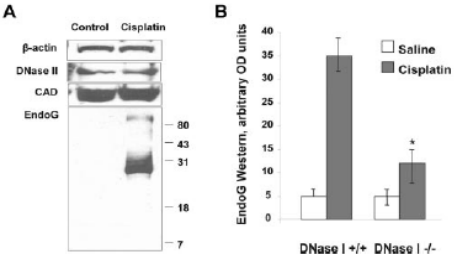

Figure 3. Induction of EndoG during cisplatin injury *in vivo*. (A) Induction of EndoG in kidney extracts measured by Western blotting during cisplatin injury in mice (4 d after a single injection with 20 mg/kg cisplatin). The significant increase of EndoG makes its presence in control kidneys almost invisible in Western. caspase-activated DNase (CAD) and DNase II are not induced. (B) Induction of renal EndoG 4 d after cisplatin injection is higher in WT than in DNase I KO mice as measured using Western and quantified by densitometry ($n = 8$; $^*P < 0.01$ between the cisplatin-treated samples).

tected by fluorescence microscopy. The immunostaining of EndoG showed that cells expressing DNase I-CRF have more EndoG, whereas cells expressing CRF have EndoG expressed at the control level (Figure 4, A and B). The expression of EndoG directly correlated with DNase I–CFP expression but not with CFP expression (Figure 4, C and D). These data suggest that DNase I is capable of inducing EndoG expression in tubular epithelial cells.

Immunohistochemical Detection of EndoG and DNA Fragmentation in Kidneys during Cisplatin Injury

Immunostaining of EndoG in normal mouse kidney showed that this enzyme is mainly located in thick limbs of the outer medulla and medullary rays (Figure 5A). In cortex, proximal tubules had the most prominent EndoG expression, whereas almost no expression was observed in glomeruli (Figure 5A, segment 1). After cisplatin injection, the amount of both EndoG and DNA fragmentation determined by TUNEL were strongly increased, and the number of tubular epithelial cell

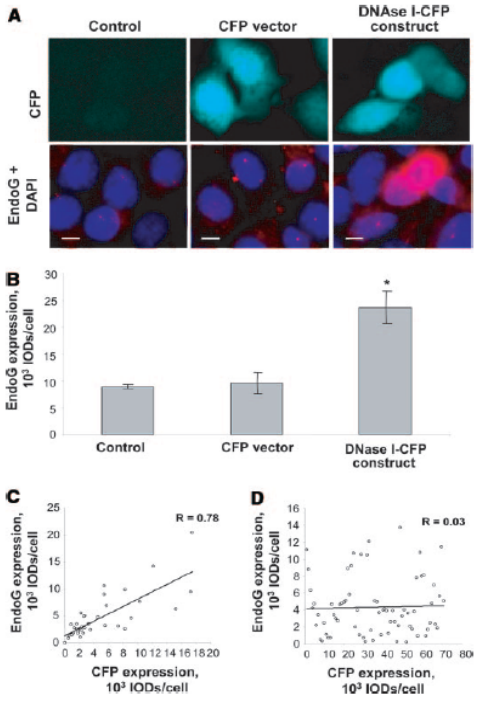

Figure 4. Induction of EndoG by DNase I in TKPTS cells. Cells were transfected with pECFP-DNaseI plasmid. The expression of CFP or DNase I-CFP fusion protein and EndoG is presented in the integrated OD units (IOD) per cell. (A) TKPTS cells expressing DNase I–CFP fusion protein but not cells expressing CFP alone show the induction of EndoG. Bars = 5 μm. (B) Quantification of EndoG expression in the transfected cells. *$P < 0.001$ *versus* either control or CFP vector. (C) Direct correlation between DNase I–CFP and EndoG expressions. (D) Absence of correlation between CFP and EndoG expressions.

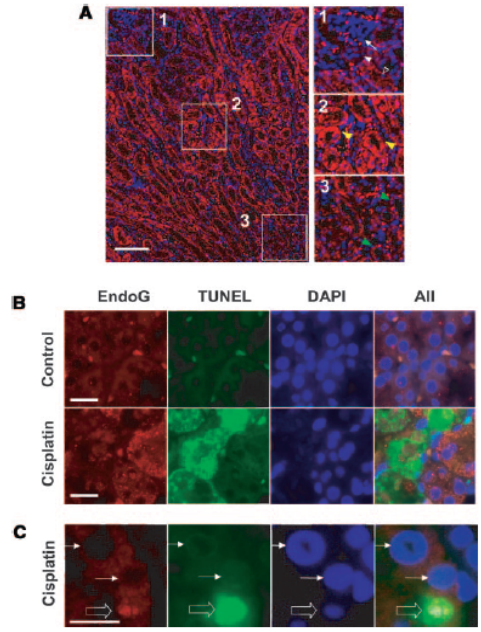

Figure 5. Immunolocalization of EndoG and DNA fragmentation in kidneys during cisplatin injury. (A) EndoG (red) is unequally distributed in the intact renal parenchyma—more in medullary rays and outer medulla (segment 2) and less in cortex (segment 1)—whereas inner medulla has the lowest expression of EndoG (segment 3). Proximal and distal tubules are shown by open and filled arrowheads, respectively. White arrow indicates glomerulus; yellow arrowheads indicate straight thick limbs, and green arrowheads indicate thin limbs. Bar = 100 μm. (B) EndoG/terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)/4′,6-diamidino-2-phenylindole (DAPI) staining of mouse kidneys 4 d after injection of saline (Control) or 20 mg/kg cisplatin (Cisplatin). Cisplatin induced the overexpression of EndoG and increased DNA fragmentation (TUNEL staining). Bar = 10 μm. (C) Nuclear translocation of EndoG is seen in mouse kidney 2 d after cisplatin injection. The nucleus shown by open arrow is stained positively for EndoG and TUNEL and has less DNA stained with DAPI. Two other nuclei above are TUNEL negative and do not contain EndoG. Bar = 5 μm.

nuclei determined by 4′,6-diamidino-2-phenylindole (DAPI) staining was decreased (Figure 5B). Some nuclei in either live or dead cells were both EndoG and TUNEL positive, indicating that nuclear import of EndoG is associated with the fragmentation of DNA and nuclei (Figure 5C).

Induction of EndoG and Suppression of DNase I Are Associated with Tubular Cell Injury by Cisplatin In Vitro

To study the cause–effect relationship between EndoG and tubular epithelial cell death induced by cisplatin, we first refined an *in vitro* model of cisplatin injury. For that, we used TKPTS cells that were treated *in vitro* with 0 to 400 μM cisplatin for 24 h. This approach revealed the dosage-dependent suppression of DNase I and induction of EndoG as determined by cell ELISA (Figure 6, A and B). These data confirmed our previous observations *in vivo* regarding the cisplatin-induced suppression of DNase I and induction of EndoG. Significant release of EndoG to cytoplasm and its import to nuclei were observed even at low concentration of cisplatin (25 μM) as determined by immunocytochemistry (Figure 6C).

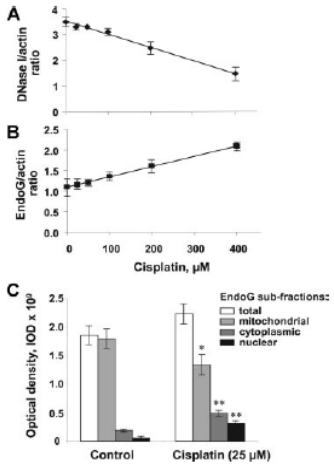

Figure 6. Dosage-dependent decrease of DNase I (A), induction of EndoG protein (B), and intracellular redistribution of EndoG (C) in TKPTS cells after cisplatin (24 h) treatment *in vitro*. (A and B) Protein expression was measured using cell ELISA as described in the Concise Methods. (C) EndoG pool redistribution measured by quantitative immunocytochemistry. Whereas the total amount of EndoG is only slightly increased after 25 μM cisplatin treatment, EndoG leaks from mitochondria and appears in cytoplasm and nuclei ($n = 3$ per group; 10 view fields per point). $^{*}P < 0.05$, $^{**}P < 0.001$ *versus* control samples.

Inactivation of EndoG Is Cytoprotective against Cisplatin In Vitro

The induction of EndoG was associated with cell death, which was measured using lactate dehydrogenase (LDH) release. To determine whether EndoG is essential for the cisplatin injury to tubular epithelial cells *in vitro*, we used two approaches. In the first, siRNA was used for EndoG silencing (Figure 7, A and B). The EndoG silencing was confirmed by the real-time RT-PCR and Western blotting. In the second, we applied the PPAR-α agonist WY-14643, which was previously shown by us to inhibit the expression of renal EndoG *in vivo*.[25] Both approaches demonstrated that the inhibition of EndoG expression is protective against cell death induced by cisplatin (Figure 7, B and C). We also attempted to determine whether inactivation of EndoG was protective against apoptosis using Annexin V/propidium iodide flow cytometry. This experiment showed that the number of Annexin V–positive apoptosis cells did not increase above 5% of total, and the correlation with EndoG silencing was absent (data not shown), suggesting that, in the used model, the inactivation of EndoG protects against necrosis, not apoptosis.

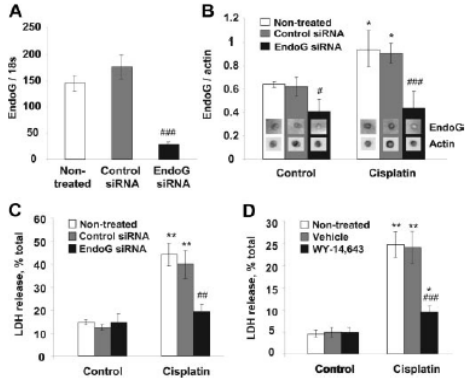

Figure 7. Protection from cisplatin injury by EndoG silencing in TKPTS cells. TKPTS cells treated with anti-EndoG small interfering (siRNA) or WY-14643 are protected from cisplatin injury *in vitro*. Inhibition of EndoG protein expression was confirmed by real-time RT-PCR (A) and Western dot-blotting (B). Cells were treated with EndoG siRNA (50 μM), control siRNA (50 μM), or transfection agent only ("nontreated") for 72 h before cisplatin (25 μM) treatment for 24 h. Total RNA was used for real-time RT-PCR, and protein extracts were used for Western blotting with anti-EndoG or anti-actin antibody. (C) Cell death after treatment with cisplatin was measured by lactate dehydrogenase (LDH) release. (D) In separate experiments, cells were grown to confluence, and medium was changed to a serum-free medium containing PBS, vehicle (DMSO), or WY-14643 (30 μM) for 2 h. Cells were then exposed to 25 μM cisplatin for 24 h, and cell death was measured by LDH release. For all graphs, $n = 3$ to 6 in each group; $^{*}P < 0.05$, $^{**}P < 0.001$ *versus* control cells without cisplatin treatment; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* nontreated cells.

DISCUSSION

Our data showed that DNase I is one of the most active endonucleases in the kidney. This observation confirmed previous reports from our and other groups.[16,17,22,23,26] DNase I was found to be highly expressed in the normal kidney along with EndoG and DNase II, whereas the expression of CAD was low. The expression of DNase γ was not observed. These data support previous observations from Shiokawa and Tanuma,[27] who reported a very low level of DNase γ expression in the rat kidney.

Inactivation of DNase I in KO mice did not affect the expression of other endonucleases. We found that despite that at the moment of cisplatin injection the activity of DNase I is higher than activity of any other endonuclease, the exposure to cisplatin strongly downregulated DNase I *in vivo* and *in vitro*. The suppression of urine DNase I (secreted by tubular epithelium) was especially profound and extended: It did not recover even at day 8. A mechanism of this phenomenon is unknown, and alternative pre-mRNA splicing can be suggested as one of the mechanisms.[19] DNase I KO mice were demonstrated in our recent study to be markedly protected against toxic injury induced by a single injection of cisplatin (20 mg/kg), by both functional and histologic criteria. *In vitro*, primary tubular epithelial cells isolated from DNase I KO mice were resistant to low-dosage (8 μM) cisplatin injury. These data provided direct evidence that DNase I is essential for kidney injury induced by cisplatin. It is interesting that the total activity of DNase I does not seem to be important for mediating cell death. It may be hypothesized that DNase I activity is already in excess and therefore deviations of activity are not as important as the presence of DNase I itself. In support of this, our latest studies of acetaminophen-induced injury to the liver, an organ with minor amounts of DNase I, showed that the inactivation of the DNase I gene is protective.[28]

Despite that DNase I was shown to be important for the cellular response to cisplatin injury, it was not clear whether it is the enzyme that produces premortem DNA fragmentation during the injury. This study demonstrated that whereas DNase I was decreased and other endonucleases remained unchanged after cisplatin treatment, EndoG was induced in both *in vitro* and *in vivo* models. We also observed EndoG translocation to nuclei at day 2 after cisplatin administration, before kidney necrosis was developed. The nuclear import of EndoG increased further with the necrosis, often observed in nuclei with fragmented DNA determined by the TUNEL assay.

Our unexpected finding was that the induction of EndoG by cisplatin was higher in WT mice than in DNase I KO mice, suggesting a positive regulatory link between the two endonucleases. The *in vitro* experiment confirmed that the overexpression of DNase I causes induction of EndoG. This places EndoG downstream of DNase I. Such a link had not been observed before and may require further investigation. We can speculate that the activation of the DNA damage pathway by DNase I–mediated DNA breaks may be one of the potential mechanisms of EndoG induction.

EndoG is a nuclease that has a unique site selectivity, initially attacking poly(dG).poly(dC) sequences in double-stranded DNA, as a result of which the enzyme got its name. EndoG resides predominantly in the mitochondria, where it is localized in the intermembrane space.[29] Mature active 27-kD EndoG can be released from mitochondria during cell death. It then translocates to nuclei and cleaves nuclear DNA without apparent sequence specificity.[20] As opposed to DNase I, this enzyme has a greater activity on single-stranded nucleic acid substrates ssDNA and RNA. It preferentially cleaves the non-canonical structures of DNA, damaged DNA, triplex DNA, and R-loops that appear during transcription.[30]

The abundance of DNase I and EndoG varies in different tissues, and kidney is one of the primary organs of their expression.[22,31] Neither DNase I nor EndoG is vitally necessary for normal tissue development. At least in some models, complete inactivation of the endonucleases did not lead to any abnormalities.[17,32] The inactivation of these enzymes in normal animals may become important if they are associated with other unknown genetic changes. This is evident from the fact that homozygous EndoG−/− knockout mice (129×C57BJ/6 background) were not viable,[33] whereas EndoG null mice, developed recently in a C57BJ/6 background, were viable.[32] Similarly, the inactivation of DNase I caused lupus only in 129×C57BJ/6 mice but not in CD-1 mice.[17,34] It was described that after cisplatin or DNase I pretreatment, DNA becomes more susceptible to EndoG digestion.[15,35]

On the basis of the finding that EndoG is downstream of DNase I and the previous demonstration of the key role of DNase I in cisplatin nephrotoxicity,[17] we tested the hypothesis that the inhibition of EndoG is cytoprotective against cisplatin injury. The two used approaches, a nonspecific inhibition of EndoG expression by WY-14643 and a specific silencing of EndoG by siRNA, showed that the inhibition of EndoG is cytoprotective against cisplatin injury *in vitro*, thus confirming the hypothesis.

Although in normal kidney cells EndoG and DNase I may be dispensable, it is clear that during cisplatin injury the presence of both endonucleases aggravates the injury. At the moment of cisplatin injection, the activity of DNase I is higher than any other endonuclease in the kidney. DNase I may introduce first ssDNA breaks after being passively translocated to nuclei as suggested by Polzar *et al.*[12] After the initial DNA damage produced by DNase I or cisplatin, DNA becomes more susceptible to EndoG digestion.[15,35] After this moment, which immediately follows the cisplatin impact, the abundance of active DNase I is very low and the rest of the DNA damage is apparently produced by EndoG. Our *in vitro* data showed that EndoG is important for cell death induced by cisplatin. Similar to previous observations in other models,[20,36] cisplatin induced the release of EndoG from mitochondria and its nuclear import. The translocation of EndoG occurred even at a low concentration of cisplatin (25 μM) that was not associated with significant induction of EndoG synthesis. Higher concentrations of cisplatin (200 to 400 μM) induced EndoG in a dos-

age-dependent manner. *In vivo*, the nuclear import of EndoG could be rarely visualized by histology, apparently because this maneuver caused prompt cell death. The presence of DNase I seems to be important only at the moment of injury, whereas variations of its activity after the initial impact by cisplatin do not matter. Further studies may determine whether this applies to other cell death endonucleases.

Taken together with the protection of DNase I KO mice against cisplatin, our data suggest that both endonucleases are essential for the tissue injury. DNase I acts immediately after cisplatin impact. Although the presence of DNase I is crucial for the kidney cisplatin injury, the activation of it does not seem to be necessary. During the inactivation of DNase I, EndoG is activated, possibly by DNase I, and it executes the DNA fragmentation, leading to the death of tubular epithelium.

CONCISE METHODS

Animals

DNase1−/− KO mice (CD-1 background) were obtained from T. Moroy (University of Essen, Essen, Germany). The mice were bred as heterozygotes and genotyped by PCR as suggested by Napirei *et al.*[34] Female 8- to 12-wk-old (20 to 30 g) mice were used in all experiments. Cisplatin (Bedford Laboratories, Bedford, OH) was administered in a single intraperitoneal injection 20 mg/kg in both *DNase1* KO and WT *DNase1* mice. Control mice were administered an injection of saline. The loss of kidney function and structural renal injury in WT mice and the absence of renal injury in *DNase1* KO mice were confirmed as described by us previously.[17] All experiments were approved by the Animal Care and Use Committee of the Central Arkansas Veterans Healthcare System.

RT-PCR

Semiquantitative RT-PCR was performed as described before.[37,38] All primers were designed to have similar reassociation characteristics and located in the coding sequence approximately 300 to 450 bp apart to allow quantification by densitometry. Primers for endonucleases were as follows: 5′-AACTCAATCGGGACAAACCT-3′ and 5′-GTTGATGTGACTGTGGTGT-3′ for DNase I, 5′-TCTGTGTGTCCCTCCCGTTC-3′ and 5′-GTCCTCTGTGGCACTGAAAG-3′ for DNase II, 5′-GCGAGGATGACTCTGTGC-3′ and 5′-CGGGGGCATTGGGCATCAC-3′ for EndoG, 5′-GCTGGCGAGACCTGGCAT-3′ and 5′-TTCTGGAGTACAGAGGCGGC-3′ for CAD, and 5′-TCACGAAGAAGCACAACATA-3′ and 5′-AAACAAACTTGGGGTCCGTC-3′ for DNase γ. All PCR products were sequenced to ensure their identity.

Real-Time RT-PCR

Our previously described protocol was followed.[39] Briefly, 1 μg of total RNA was reverse-transcribed in a 50-μl reaction followed by real-time RT-PCR in a 25-μl reaction using SmartCycler (Cepheid, Sunnyvale, CA). Reaction mix was prepared using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Two-temperature cycles with annealing/extension temperature at 62°C for EndoG and 64°C for 18s were used. The fluorescence was measured at the end of the annealing step. The melting curve analyses were performed at the end of the reaction (after 45th cycle) between 60 and 95°C to assess the quality of the final PCR products. The threshold cycle (Ct) values were calculated by fixing the basal fluorescence at 15 units. cDNA samples were diluted for real time 1:5 and 1:200 for EndoG and 18s respectively. Three replicate reactions were performed for each sample, and the average Ct was calculated. The standard curve of the reaction effectiveness was performed using the serially diluted (5 points) mixture of all experimental cDNA samples for EndoG and 18s separately. Calculation of the relative RNA concentration was performed using Cepheid SmartCycle software (version 2.0d, Sunnyvale, CA). Data are presented as ratio of EndoG/18s mRNA.

Total Endonuclease and DNase I Activities

Total kidney extracts were prepared, and endonuclease activity was measured by the plasmid incision assay using pBR322 plasmid (New England Biolabs, Beverly, MA) as substrate as described previously.[18] The reaction was carried out in 2 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), and 0.5 mM dithiothreitol to determine the Ca/Mg-dependent endonuclease activity or in 5 mM $MnCl_2$, 10 mM Tris-HCl (pH 7.4), and 0.5 mM dithiothreitol for the Mn-dependent activity. One unit of endonuclease was capable of converting 1 μg of covalently closed supercoiled plasmid DNA to open circular or linear isoforms in 1 h at 37°C. Protein was measured using the BCA protein assay (Pierce, Rockford, IL). BSA was used as standard. DNase I activity was determined using the single radial enzyme diffusion assay[40] or by the zymogram gel electrophoresis of total protein extracts as described by us previously.[18]

Western Blotting

Total kidney extracts were prepared as described previously.[18] Proteins (10 to 20 μg) were separated in 11.5% gel according to the Laemmli procedure.[41] Electrophoresis was performed at 100 V for 2 h. Proteins were transferred to the nitrocellulose membrane in Novex transferring buffer (Invitrogen) at 40 V for 3 h. For dot-blotting, the electrophoresis step was omitted and the samples were applied directly to membrane (0.25 μg protein/dot). After soaking in the blocking solution overnight at 4°C, the membrane was incubated with polyclonal anti-EndoG (Chemicon, Temecula, CA) or other antibodies diluted 1:1000 and washed in Tris-buffered saline, and primary antibodies were detected with anti-rabbit IgG horseradish peroxidase (HRP) using a SuperSignal chemiluminescent kit (Pierce). For allowing quantification, filters were stripped with 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris (pH 6.7) at 50°C and rehybridized with anti-actin 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA). OD of bands was determined by densitometry in quadruplicate format and normalized by actin.

Immunohistochemistry and TUNEL Assay

Samples of kidney tissue (1.5 mm thick) were fixed with 10% neutral formalin (Sigma, St. Louis, MO) for 24 h, dehydrated, and embedded in paraffin. For EndoG, TUNEL, and DAPI staining, sections of 3 μm thickness were cut, dewaxed, rehydrated in PBS (10 mM sodium phosphate buffer [pH 7.4] and 140 mM NaCl), pretreated with pro-

teinase K (20 μg/ml) for 20 min at 37°C, and probed with diluted 1:400 anti-EndoG antibody (Chemicon) in blocking buffer (1% BSA [wt/vol], 0.012% saponin [wt/vol], and PBS) at 4°C overnight. The primary antibody was detected the next day after triple washing with 0.05% Tween-20 in PBS (PBST) by probing with 5 μg/ml solution in the same blocking buffer of anti-rabbit Ig (IgG; goat) conjugated with AlexaFluor 594 (Molecular Probes, Eugene, OR) for 30 min at 37°C, and after the washing, sections were then analyzed with the TUNEL assay using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN). TUNEL were performed according to the manufacturer's protocol. Each section was probed with a reaction mixture of terminal deoxynucleotidyl transferase and anti-actin–FITC–labeled precursor in cacodylate-based buffer for 1 h at 37°C, rinsed with PBST twice and water once, and counterstained with 10 μM DAPI (Sigma) for 5 min. The slides were washed with water three times, mounted under a ProLong Antifade Kit (Molecular Probes), and analyzed using a Carl Zeiss microscope with ×10, ×40, and ×100 objectives. Controls of the specific reaction of anti-EndoG antibody and nick-labeling were performed with substitution of the reagents with the buffers.

Cell Culture

TKPTS cells were obtained from Dr. Elsa Bello-Reuss (University of Texas Medical Branch, Galveston, TX) and were cultured as described previously.[42] The cells were maintained in DMEM/Ham F-12 medium (Sigma) supplemented with 7% FBS (Hyclone, Logan, UT). Cells were maintained in a humidified incubator gassed with 95% air/5% CO_2 at 37°C, fed at intervals of 48 to 72 h, and used within 1 d after confluence (except for the siRNA experiments described in the EndoG siRNA Silencing section).

Construction of Expression Vector and Transfection

Previously cloned DNase I gene[19] was inserted in pECFP-N1 vector (Clontech, Mountain View, CA) upstream of the gene encoding CFP. TKPTS cells were transfected using Lipofectamine 2000 (Invitrogen), and the expression of DNase I–CFP fusion protein or CFP alone (control) was detected by fluorescence microscopy using a cyan filter.

EndoG siRNA Silencing

TKPTS cells were seeded in six- or 24-well plates and grown to 60 to 70% confluence. To knockdown EndoG mRNA, cells were transfected with siRNA duplexes (sense siRNA 5′-AUGCCUGGAACAACCUUGAdTdT-3′ and antisense siRNA 5′-UCAAGGUUGUUCCAGGCAUdTdT-3′) or control siRNA #1 (Dharmacon, Lafayette, CO). The cells were treated with 50 nM siRNA mixed with TransIT-TKO transfection reagent (Mirus, Houston, TX) according to the manufacturer's recommendations, in serum-free medium for at least 48h. After that, the transfection medium was removed and the cells were treated with cisplatin for 24 h. EndoG mRNA expression was measured using real-time RT-PCR.

Cell ELISA

Cell ELISA was performed as described by Frahm *et al.*[43] The cells were seeded in a 96-well plate (10,000 cells/well) and grown in a complete medium for 24 h. The cells were washed with serum-free medium, permeabilized, and fixed with fixative (4% wt/vol paraformaldehyde, 0.012% saponin, and PBS) for 10 min at room temperature. After fixation, the cells were washed and rehydrated in PBS and the endogenous peroxidase activity was inhibited by exposure to 0.5% hydrogen peroxide for 30 min at room temperature. Then the cells were probed with the primary antibody in blocking buffer (2% BSA and PBS) for 2 h. After triple washing with PBST (0.05% Tween-20 and PBS), primary antibody (titers 1:500 to 1:1000) was detected with anti-rabbit antibody (Santa Cruz) conjugated with HRP. The HRP activity was measured with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma) at 450 to 540 nm using the Synergy HT-I microplate reader (Bio-Tek Instruments, Winooski, VT). After measurement and triple washing, wells were reprobed with anti–actin-FITC antibody (titer 1:10; Santa Cruz Biotechnology) and washed again, and the fluorescence was measured at 485/528 nm (excitation/emission). All measurements were done in quadruplicate per one marker per one cell line and were repeated at least three times in different plates. Negative controls of primary antibody were done by their substitution by blocking buffer.

Measurement of EndoG in Cellular Compartments

TKPTS cells that were treated with cisplatin or vehicle were washed twice with PBS and fixed with 4% paraformaldehyde and 0.12% saponin in PBS for 10 min. Cells were probed with rabbit anti-EndoG (1:400) and mouse anti-cyclooxygenase IV (1:200) antibodies, which were detected with goat anti-rabbit AlexaFluor 594 and goat anti-mouse AlexaFluor 488 conjugates, respectively. Nuclei were counterstained with DAPI. Slides were analyzed under magnification of 600 using an Olympus IX-81 microscope (Olympus America, Center Valley, PA). Images and acquisitions were made with a digital camera HAMAMATSU ORCA-ER (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and software Slidebook 4.1 (SciTech Pty Ltd., Preston, Victoria, Australia). OD of cyclooxygenase IV signal was used as the marker of mitochondrion localization and subtracted from the EndoG optical signal. The result was considered "nonmitochondrial EndoG," and the rest of the signal was considered "mitochondrial EndoG." For measurement of EndoG presence in nuclei, images were traced by DAPI, and part of nonmitochondrial EndoG, which was co-localized with DAPI, was considered nuclear EndoG.

Cytotoxicity Assays

For measurement of cytotoxicity of cisplatin, the LDH release assay kit (Promega, Madison, WI) was used. The results were expressed as the ratio of LDH released by treated cells into medium to the total LDH. In the Annexin V/propidium iodide assay, Annexin V-FITC (Invitrogen) and propidium iodide (Sigma) were used as described by us previously.[44]

Statistical Analyses

Results were expressed as means ± SEM. The significance of difference in mean values within and between multiple groups was examined with an ANOVA for repeated measures followed by a Duncan's *post hoc* test. The *t* test was used to evaluate the significance of differences between two groups of experiments (SigmaStat; SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

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DISCLOSURES

None.

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