Establishment of a Mouse Macula Densa Cell Line with an nNOS Promoter Driving EGFP Expression

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Abstract: We describe a unique method for establishing a functionally intact macula densa cell line from immortalized renal cells in culture. The macula densa is involved in the tubuloglomerular feedback (TGF) system in the kidney and specifically expresses neuronal nitric oxide synthase (nNOS). A 347 bp portion of the nNOS promoter was used to drive the expression of enhanced green fluorescence protein (EGFP). An immortalized distal tubule (DT) cell line was derived from distal tubules microdissected from the kidneys of SV40 large T antigen transgenic

mice. Immunofluorescence labeling using an antibody against nNOS revealed no specific EGFP expression in immunofluorescence-negative DT cells. The established cell line (NE-MD) showed a time-dependent increase in signals of the nNOS protein when they were incubated with 12 μ M furosemide (an inhibitor of Na^+ - K^+ -2 Cl^- symporter) for 5 h. In conclusion, this newly developed macula densa cell line will be useful in studies of the TGF system. [The Japanese Journal of Physiology 55: 365–372, 2005]

Key words: macula densa, neuronal nitric oxide synthase (nNOS), mouse kidney, furosemide, EGFP.

The macula densa (MD) is a plaque of specialized tubular cells within the end portion of the cortical thick ascending limb (cTAL), which is found in the angles between the afferent and efferent arterioles of the corresponding glomerulus [1]. MD cells monitor changes in the tubular NaCl concentration, whereby they regulate the glomerular filtration rate (GFR) by changing the sizes of the afferent and efferent arterioles. This negative feedback loop, known as the tubuloglomerular feedback (TGF) system, controls renal hemodynamics and extracellular fluid volume [2–5]. The microdissected juxtaglomerular apparatus (JGA), including MD cells, may be highly useful *in vitro* for studying the physiological and biochemical factors of the TGF system [6, 7]. However, freshly isolated MD cells, as well as the JGA, are not readily available because of their anatomical location in the kidney.

Therefore, the establishment of a highly differentiated MD cell line is of considerable importance because a supply of cultured MD cells would allow a powerful expansion of basic investigation. Yang *et al.* [8] have recently generated a mouse macula densa cell line, MMDD1, which was developed from transgenic mice for the SV40 large T-antigen (6-TgN(SV)7Bri) by using sorting methods with FITC and phycoerythrin fluorescence. However, MMDD1 cells resembling macula densa may not be genetically pure because they express NKCC1 [9], an isoform of NKCC2 (a Na^+ - K^+ -2 Cl^- symporter [10]), localized in the inner medullary collecting duct (IMCD) [11].

nNOS is expressed in diverse cell types and tissues, such as skeletal muscle [12], pancreatic β cells [13], and kidneys [14, 15]. The finding that unique promoters participate in nNOS expression in unique cell types

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suggests that the expression of nNOS mRNA in tissues such as skeletal muscle and macula densa is subject to tissue-specific regulatory pathways [16]. This opens the possibility that MD cells can be visualized with the expression of enhanced green fluorescence protein (EGFP) and cloned from the primary culture of kidney distal tubule cells [15].

We first established immortalized renal tubular cell lines from transgenic mice harboring SV40 large T-antigen gene [17] by microdissecting distal tubules [18, 19]. We then developed a stable cell line expressing EGFP under the control of the nNOS promoter. The nNOS (*NOS1*) isoforms are expressed from a human genomic locus spanning 240 kb at 12q24.2 [20]. At least nine unique variants express in the noncoding exon 1 of nNOS mRNA [21]. Each exon 1 variant is tissue-specifically expressed [21], suggesting that the expression (of these variants) may be controlled by each tissue/cell-specific promoter located immediately upstream of each variant. In the present study, we used a promoter region of 347 bp immediately upstream of the exon 1c, which is reported to be preferentially active in the kidney [22]. Finally, we have successfully established a macula densa cell line with an nNOS-EGFP transgene (NE-MD). NE-MD cells exhibit unique properties of furosemide induction of nNOS protein expression.

MATERIALS AND METHODS

Materials. Collagenase (type I), G 418, bovine serum albumin (BSA), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and leupeptin were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Lipofectamine™, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin/EDTA were from Invitrogen-Life Technologies (Carlsbad, CA, USA). Human transferrin was from Roche Diagnostics GmbH (Mannheim, Germany). RITC80-7 medium was from Kyokuto Pharmaceutical Industrial (Tokyo). Insulin was from Nordisk Pharmacy (Tokyo). Recombinant human epidermal growth factor (rhEGF) was from Wakunaga Seiyaku (Tokyo). Dulbecco's modified Eagle's medium (DMEM) was from Nissui Seiyaku (Tokyo). Pentobarbital sodium salt was from Dainihon-seiyaku (Osaka). All other chemicals were of analytical grade from Waco Pure Chemical (Osaka). Polyvinylidene difluoride (PVDF) membrane and ECL kits were from Amersham Biosciences UK Limited (Little Chalfont Buckinghamshire, UK). Primary antibodies: anti-nNOS antibodies (Cayman Chemical Co., Ann Arbor, MI, USA) and mouse anti-actin monoclonal antibody

(Chemicon International Inc, Temecula, CA, USA). Secondary antibodies: A horseradish peroxidase-conjugated goat antirabbit or goat antimouse antibody (Amersham Biosciences).

Transgenic mouse strain. The origins and characteristics of SV40 large T-antigen expressing transgenic mice, provided by Dai-ichi Seiyaku (Tokyo), have been described elsewhere [17]. In brief, the immortalized cell lines were rapidly established from the primary culture of different tissues of this strain [17, 19]. The established cell lines grew at a permissive temperature (33°C), but not at a nonpermissive temperature (39°C). The experimental protocol abided by the guidelines (1998) of the Kitasato Medical Experimental Animal Care Committee.

Distal tubule isolation and culture techniques. Distal tubules (DT) containing MD cells were microdissected from slices of kidney cortex according to the method described in previous papers [18, 19]. Briefly, each mouse was anesthetized with pentobarbital sodium salt (37.5 mg/kg body wt, I.P.) and was perfused through the heart with a dissociation solution, Dulbecco's modified Eagle's medium (DMEM), containing 1 mg/ml of collagenase type I. The cortex slices (approximately 1 mm thick) were incubated in the same dissociation solution for 30 min at 33°C. The slices were rinsed twice and the distal tubules microdissected in Hanks' balanced salt solution (HBSS), containing 0.1% BSA, at 4°C. The distal tubule (including the end portion of the cortical thick ascending limb of Henle's loop) was identified by its attachment to an original glomerulus [19]. The dissected distal tubules were maintained in RITC80-7 medium at 33°C (5% CO₂/95% air). Media were supplemented with 5% FBS, 10 µg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml rhEGF, 50 U/ml penicillin, and 50 µg/ml streptomycin. When the DT cells became confluent after 7–10 days, they were maintained by subculture. The cells were used for experiments from passages 10 to 20.

PCR. DNA fragments containing 5' and 3' deletions of the promoter region (347 bp) immediately upstream of exon 1c of human nNOS gene (GenBank Accession No. AJ308545) [22] were generated by PCR. Details of the 347 bp are available upon request. Human genomic DNA (Cat. No. 6550-1; BD Biosciences [Clontech], Palo Alto, CA) was amplified by using a 279S forward primer and a 49AS reverse primer. They correspond to nucleotides 5'-CAG ACG CTG CCA CTG CTG-3' and 5'-GTC ACC CCC TCT CAG ACA GTG-3' of human nNOS gene, respectively. PCR was per-

Table 1. Primers used for RT-PCR.

Gene product	sequence (5'-3')	PCR product (bp)	Annealing temp. (°C)	References
G-6-Pase	GAA CGC CTT CTA TGT CCT CT CAC CGG AAT CCA TAC GTT GA	300	60	8
THP	CTG GAT GTC CAT` AGT GAC TC TGT GGC ATA GCA GTT GGT CA	400	57	8
ROMK	AGA AGA GGT GCT TTG GGG TT GAT GCT TCT GAA CAC AGC CA	313	60	26
NCC	AAT GGC AAG GTC AAG TCG TGA TGC GGA TGT CAT TGA TGG	205	62	27
nNOS	ATA CCA GCC TGA TCC ATG GA CTC CAG GAG GGT GTC CAC	591	58	28
COX-2	ACT CAC TCA GTT TGT TGA GTC ATT TTT GAT TAG TAC TGT AGG GTT ATT	583	58	29
NKCC1	CAG AGA CAA TTT GAA GAC C CCA ACG TCA GCA TGA GAT	681	58	9
NKCC2	CCC GCT CTC TTG GAT ATA TAA C GCT CCG AAC AAA TTC TTC CG	509	60	9

formed in a reaction mixture (100 µl) that contained 1× PCR buffer, 2.5 mM dNTPs, 100 pmol of primers, 2.5 µl of the human genomic DNA as a template, and 2.5 U of ExTaq DNA polymerase (Takara-Bio, Ohtsu) [23]. PCR conditions were 95°C, 5 min (incubation) and 30 cycles 95°C, 1 min (denaturation); 60°C, 1 min (annealing); and 72°C, 2 min (extension) and finally 72°C, 7 min. Unless otherwise indicated, all reactions were carried out at room temperature (26–28°C).

DNA sequencing. The PCR product was isolated by the Wizard PCR Preps DNA purification system (Promega Co., Madison, WI, USA) and cloned into a pGEM-T Easy vector (Promega Co.), pGEM-T-N. The insert of pGEM-T-N was subjected to DNA sequence analysis by using an ABI Prism 3700 capillary sequencer (Applied Biosystems, Foster City, CA, USA).

Construction of the nNOS promoter-driven expression vector. The nNOS promoter sequence (347 bp) was excised from the vector by the digestion of pGEM-T-N with *EcoRI* and blunted and subcloned into the *SalI*–*BamHI* sites of pCE-29 [24, 25], yielding nNOS promoter-EGFP (pNE). An expression vector pNE-EGFP-1 was constructed by cloning the fragment from pNE in the *SalI*–*SacI* sites (1.7 kb) into the same sites of the promoterless EGFP vector, pEGFP-1 (Cat. No. 6086-1; BD Biosciences [Clontech]), to introduce the neomycin cassette.

Cloning of cells carrying the pNE-EGFP-1 vector. The DT cells were transfected by using linearized pNE-EGFP-1 (2 µg) with *SalI* and 4 µl of Lipofectamine™ Reagent (Invitrogen) per 35 mm dish.

The cells were incubated in G418-free RITC80-7 with 5% FBS at 33°C for 24 h and maintained in RITC80-7 containing 0.8 mg/ml of G418. Media were changed every three days. Ten days after transfection, the clonal cells (G418-resistant cells expressing EGFP) were visualized under fluorescent microscopy (wavelength 530 nm) and marked on the bottom of a culture dish. The fluorescent cells/colonies were mechanically peeled off and collected with a sterile yellow tip (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Collected cells expressing EGFP were gently trypsinized, washed, and sparsely plated in new 35 mm plastic dishes containing RITC80-7 medium with G418. Three or four days after seeding, the fluorescent colonies were again collected and plated. Finally, all the cells in a dish expressed stable EGFP. These fluorescent cell lines were named “NE-MD,” since the clonal cells derived from DT cells carried the NE transgene in their own chromosomes.

RT-PCR. Total RNA was prepared from cultured NE-MD cells by using the Rneasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The positive control RNA used was Mouse Kidney Total RNA (BD Biosciences). One microgram of total RNA was used for reverse transcription with an RNA PCR kit (AMV), ver. 3.0 (Takara-Bio). The products were separated on Tris-acetate/EDTA 2% agarose gels containing 100 ng/ml ethidium bromide. Sequences of all primers used in this study and the expected sizes of the amplification products are summarized in Table 1 [8, 9, 26–29]. ROMK (renal outer medullary

Fig. 1. Photomicrographs of phase-contrast (A, C, E) and fluorescence microscopy (B, D, F). DT cells transfected with pNE-EGFP-1 (2 days) (A, B). EGFP-positive and EGFP-negative colonies are, respectively, indicated by the single

and double arrows (7 days) (C, D). The establishment of nNOS-EGFP positive cells (10 days) (E, F). Note that all cells are EGFP-positive. The culture media contained G418 for selection. Bars indicate 200 μ m.

potassium channel) mRNAs were widely expressed in the distal nephron, such as thick ascending limb and distal convoluted tubule [30]. Further, the immunoblot signals (80 kDa) of anti-ROMK were equally stained between DT and NE-MD cells (unpublished data). Thus ROMK mRNA was used as an internal standard [31].

Immunoblot analysis. Immunoblot analysis was performed according to standard techniques [32, 33]. Transfected and nontransfected cells were incubated in lysis buffer (TBS containing [mM] 24 Tris, 136 NaCl, 2.6 KCl, and leupeptin, pH 7.4). The homogenates were centrifuged at 800 rpm for 5 min (4°C) to remove cell debris. Supernatants were diluted in (SDS) sample buffer with 6% β -mercaptoethanol. The proteins were denatured in boiling water for 10 min, separated by polyacrylamide gel electrophoresis, and transferred to a (PVDF membrane). The blots were blocked for 30 min with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS/T), followed by incubation overnight at 4°C with anti-nNOS antibodies (diluted 1:10,000) and mouse antiactin monoclonal antibody (diluted 1:500). After washing with TBS/T, the blots were incubated with a horseradish peroxidase-conjugated goat antirabbit or goat antimouse secondary antibody for 2 h. They were then washed with TBS/T and subjected to visualization with the use of ECL kits according to the manufacturer's instructions.

RESULTS

Transfection with linearized pNE-EGFP-1

DT cells were transfected with a linearized pNE-EGFP-1 vector encapsulated by Lipofectamine (Invitrogen), then cultured in the presence of G418 for at least 10 days. Two days after transfection, some clones exhibited bright EGFP, but others did not (Fig. 1, A and B). EGFP-expressing clones (Fig. 1, C and D) were mechanically peeled off and collected with a yellow tip under sterile conditions (see METHODS). This procedure was repeated several times until all cells of the clone expressed EGFP powerfully (Fig. 1, E and F). We finally established four clones, which were named the "NE-MD" cell line, nNOS-EGFP expressing macula densa. Confluent NE-MD cells have an epithelial, cobblestone morphology appearance on phase contrast light microscopy (Fig. 1E). EGFP-expressing cells look spindly under the same conditions (Fig. 1, C and D). One cell line of the NE-MD clones that strongly expresses EGFP has been characterized for the present study.

Characterization of NE-MD cells

The cell line used in the present study expresses mRNAs of specific markers for macula densa, including nNOS, NKCC2, and COX-2 (Fig. 2). It also expresses the renal outer medullary potassium channel

Fig. 2. RT-PCR analysis of total RNA from NE-MD cells and kidney. Characterization of NE-MD cells by RT-PCR of G-6-Pase, THP, ROMK, NCC, nNOS, COX-2, NKCC1, and NKCC2. Lane 1: NE-MD cells; lane 2: kidney cells. Kidney RNA was used as a positive control. ROMK was used as an internal standard.

(ROMK) mRNA, but does not express the mRNA for NKCC1 (Fig. 2). Moreover, glucose-6-phosphatase (G-6-Pase), Tamm-Horsfall protein (THP), and the thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter (NCC), respectively, used as a marker for the proximal tubules, for the thick ascending limb of Henle (TAL), and for the distal convoluted tubule (DCT), were negative in the NE-MD cell line (Fig. 2).

Induction of nNOS by furosemide

The NE-MD and DT cells both were incubated with 12 μM furosemide, an inhibitor of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ transporter (NKCC2), for 0.5, 1, 2, and 5 h. Cell lysates of both cell lines were isolated at each time point and subjected to immunoblot analysis by the use of anti-nNOS antibodies. Signals of the nNOS proteins in NE-MD cells increased significantly in a time-dependent manner, but no increase was observed in the DT cells (Fig. 3).

DISCUSSION

By utilizing the nNOS promoter-driven expression of EGFP, we established a unique macula densa cell line (NE-MD) from cultured kidney distal tubule (DT) cells obtained from SV40 large T-antigen transgenic mice. In the present study, a vector carrying the nNOS promoter and EGFP cDNA was successfully introduced into the genome of DT cells. The macula densa cell line “NE-MD” was established, using a promoter region (347 bp) immediately upstream of the exon 1c of nNOS [22], and therefore it is unique and completely different from MMDD1, mouse kidney macula densa cells [8].

The molecular mechanisms underlying tissue (cell type)-specific expression and the diverse sizes of nNOS proteins appear to be the utilization of alternative promoters [16]. Major bands in brain and skeletal muscle, respectively, have been found at approximately 155 kDa and 164 kDa [34]. In MMDD1 [8] and NE-MD cells, the proteins were stained strongly at approximately 100 and faintly at about 65 kDa. Since the nNOS gene is single, it is probable that isoforms of the nNOS proteins are generated by each of the different tissue/cell-specific promoters [22]. In the kidney, at least three kinds of nNOS mRNA (carrying sequences corresponding to the exons 1c, 1f, and 1g of the nNOS gene at their 5' end) are expressed [22]. Therefore the expression of these mRNA may be regulated by each of the promoters upstream of the noncoding 1st exons. It is not unusual that nNOS molecules would differ in molecular size in the kidney and skeletal muscle. In the present study, we used a 347 bp promoter region immediately upstream of exon 1c of the nNOS gene to express EGFP in DT cells, and nNOS in a skeletal muscle has an additional 34 amino acid insert, encoded by 102 bp sequences, between the calmodulin-binding site and the flavin mononucleotide (FMN)-binding site of nNOS [34].

Fig. 3. Representative immunoblots of protein extracts of NE-MD and DT cell lines after furosemide treatment. Quiescent kidney cells were stimulated with 12 μM furosemide. Proteins were extracted from NE-MD or DT cells at 0, 0.5, 1, 2, and 5 h after treatment with furosemide and subjected to immunoblotting, using antibodies against nNOS and actin.

NE-MD cells, which originate from cultured DT cells, express mRNAs of specific markers for the macula densa, including nNOS [28], NKCC2 [8], and COX-2 [29]. Furthermore, they do not express markers for other nephron segments, such as G-6-Pase for the proximal tubules [9], THP for TAL with the exception of the macula densa [8], and NCC for DCT [27]. Thus we believe that a cell line of NE-MD is a valid model of the kidney macula densa. There is no difference between NE-MD and MMDD1 [8, 9] in terms of the expression patterns of G-6-Pase, THP, nNOS, COX-2, ROMK [35, 36], and NKCC2. However, the staining of NKCC1 mRNA was different between the two cell lines. NKCC1 mRNA was not detectable in NE-MD in the present study, whereas it is strongly positive in MMDD1 cells [9]. This result is consistent with the reported properties of the macula densa of the mouse kidney, in which the NKCC1 (BSC2) protein is not detectable. The NKCC1 protein is localized in the terminal IMCD [11].

This is the first reported observation that nNOS expression in cultured macula densa cells (NE-MD) is enhanced by an application of furosemide. Immunoblot analysis in the present study revealed a staining of the nNOS protein (65 kDa) to be faint, but significant, in NE-MD cells, though practically no detectable signal was observed in DT cells. More important, signals of nNOS increased time-dependently when NE-MD cells were incubated with 12 μ M furosemide, an inhibitor of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ symporter. These findings may be consistent with the regulation of nNOS *in vivo* by salt restriction [28, 37, 38] as well as an application of angiotensin II [39] or furosemide [40]; however, the cellular mechanism remains unclear. The furosemide-induced nNOS expression in rat kidney [40] and cultured NE-MD cells in the present study is apparently different from previous observations that furosemide activates renin synthesis on afferent arterioles freshly isolated from the rat kidney [41], and that a generation of NO occurs only at elevated levels of $[\text{NaCl}]_i$ [42]. Further, nNOS activity in the macula densa is reported to be stimulated by the increased intracellular pH [43] caused by alterations of luminal NaCl concentration [44]. However, these functional studies interrelate macula densa luminal NaCl uptake with nNOS activity and do not rule out the possibility that the expression of nNOS protein or mRNA is increased by furosemide. Thus it is perhaps not surprising that the expression of nNOS is regulated at various levels, including the genomic pathway [39], though the expression of nNOS (NOS I, bNOS) is constitutive [8, 14, 15, 37, 45, 46].

In conclusion, the NE-MD cell line established in the present study should also prove helpful in assessing these issues.

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