Glucose Induces Clonal Selection and Reversible Dinucleotide Repeat Expansion in Mesangial Cells Isolated from Glomerulosclerosis-Prone Mice

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Clonal selection has been proposed as a pathogenetic mechanism in various chronic diseases, such as scleroderma, hypertension, pulmonary fibrosis, interstitial fibrosis of the kidney, atherosclerosis, and uterine leiomyomatosis. We previously found that mesangial cells from ROP mice prone to develop glomerulosclerosis changed their phenotype in response to high glucose concentrations. Here, we investigate whether clonal selection might contribute to this phenotype change. We found that in ROP mice at least two distinct mesangial cell clones exist. They are characterized by a different length of the d(CA) repeat in the MMP-9 promoter and exhibit a significantly different gene expression profile. Exposure of ROP mesangial cells to 25 mmol/l glucose for 35 days induces both clonal selection and reversible dinucleotide repeat expansion. None of these findings were present in mesangial cells isolated from C57BL/6 mice, which are not sclerosis-prone. We conclude that mesangial cell michrochimerism may be a marker for the susceptibility to glomerulosclerosis, that dinucleotide repeat expansion may be a novel mechanism for glucose-induced changes in gene expression, and that clonal selection may partially explain the change in mesangial cell phenotype in diabetes. *Diabetes* 52: 2594–2602, 2003

Extracellular matrix accumulation leading to progressive glomerulosclerosis is one of the hallmarks of diabetic nephropathy, and mesangial cells have been shown to be the major source of extracellular matrix proteins in glomerulosclerotic lesions (1). It is interesting that mesangial cells isolated from diabetic mice exhibit a distinctly different phenotype in vitro when compared with mesangial cells isolated from age-matched nondiabetic mice of the same strain (2–5). Similarly, skin fibroblasts isolated from patients with diabetes undergo a phenotypic change (6–8). Comparable observations have been made in skin fibroblasts from patients with scleroderma (9,10) and vascular smooth muscle cells isolated from patients with hypertension (11).

This has led to the hypothesis that mesangial cells in vivo change their phenotype in response to the diabetic environment. The underlying pathogenetic mechanisms are not yet understood. Clonal selection has been proposed as a pathogenetic mechanism in various chronic diseases, such as scleroderma (9,10), hypertension (11), pulmonary fibrosis (12), interstitial fibrosis of the kidney (13,14), atherosclerosis (15), and uterine leiomyomatosis (16). It has been shown that clonal patches of smooth muscle cells exist in normal vessel walls and that a clone with specific properties may give rise to atherosclerotic plaques (17,18). The selection of an mesangial cell clone with the "disease phenotype" in an individual with diabetes may represent one of the mechanisms leading to the change in phenotype in mesangial cells isolated from individuals with diabetes.

We have previously identified two mouse strains, ROP and C57BL/6 that can be used as models that mimic the propensity or resistance to develop glomerulosclerosis in patients (19,20). In addition, we showed that mesangial cells isolated from these strains maintained a distinctly different phenotype in vitro and responded differently to glucose (21). Thus, we used mesangial cells isolated from ROP and C57BL/6 mice to test the hypothesis that in mice susceptible to developing diabetic nephropathy, mesangial cell clones with a distinctly different phenotype and genotype exist and that exposure to 25 mmol/l glucose in vitro and diabetes in vivo leads to a clonal selection of mesangial cell subpopulations.

RESEARCH DESIGN AND METHODS

Cell culture and single-cell clones. Mesangial cells were isolated from four ROP.B6 (ROP) and three C57BL/6 mice (B6; all obtained from The Jackson Laboratory, Bar Harbor, ME) and characterized as previously described (21). In addition, 100 mesangial cell clones were generated by dilution from each uncloned mesangial cell population and recharacterized for mesangial cell markers. For certain experiments, mesangial cell subclones were generated from mesangial cell clones. Mesangial cells were cultured in 6 mmol/l glucose media, exposed to 25 mmol/l glucose for 35 days, and then returned to baseline conditions for another 35 days as previously described (21). Mesangial cells propagated in 6 mmol/l glucose for the entire period of 70 days served as controls. Media containing 6 mmol/l glucose were supplemented with 19 mmol/l mannitol to account for the different osmolarities of high- and low-glucose media. The cells were trypsinized weekly under each condition and collected for PCR analysis. Mesangial cell subclones were used to determine whether spontaneous mutations occurred. We generated 100 single-cell subclones derived from two mesangial cell clones with 20 d(CA) repeats. The repeat length was verified by PCR. Mesangial cell subclones were then exposed to 25 mmol/l glucose for 35 days. The d(CA) repeat length was checked by PCR in each subclone after 10 and 35 days of exposure to 25 mmol/l glucose. Because every subclone had been derived from a single cell with 20 d(CA) repeats, any change in d(CA) repeat length occurring during the
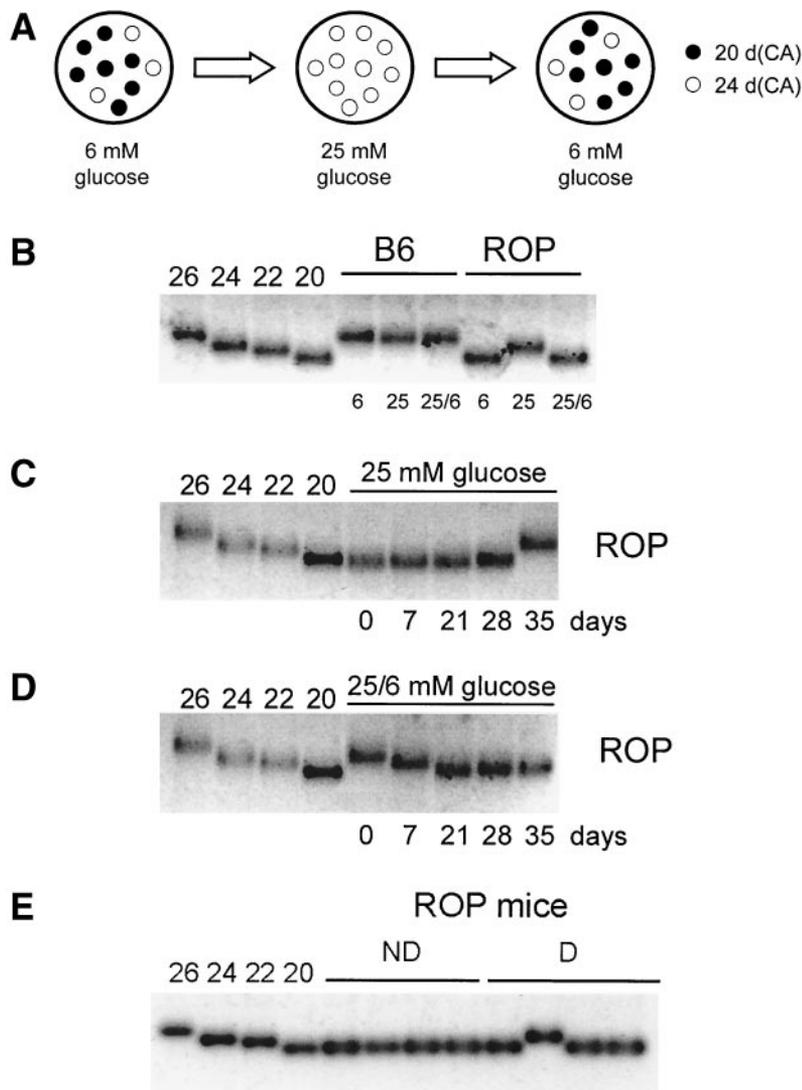
A

6 mM glucose → 25 mM glucose → 6 mM glucose

● 20 d(CA)
○ 24 d(CA)

B

26 24 22 20 | B6 | ROP

6 25 25/6 6 25 25/6

C

26 24 22 20 | 25 mM glucose

ROP

0 7 21 28 35 days

D

26 24 22 20 | 25/6 mM glucose

ROP

0 7 21 28 35 days

E

ROP mice

26 24 22 20 | ND | D

FIG. 1. Glucose and diabetes induce a change in the d(CA) repeat length in the MMP-9 promoter. *A*: Schematic representation of the experimental design. Mesangial cells cultured in 6 mmol/l glucose were exposed to 25 mmol/l glucose for 35 days. The glucose concentration was then restored to 6 mmol/l for another 35 days. *B*: Representative PCR for the MMP-9 promoter region containing d(CA) repeats in B6 and ROP mesangial cells cultured in 6 mmol/l glucose (6), exposed to 25 mmol/l glucose for 35 days (25), and returned to 6 mmol/l glucose for 35 days (25/6). There was no change in B6 mesangial cells, whereas the d(CA) repeat length in ROP mesangial cells changed from 20 to 24 and back to 20 d(CA) repeats. *C*: Representative PCR showing the time required for induction of the glucose effect in ROP mesangial cells. A change in d(CA) repeat length from 20 to 24 d(CA) repeats was observed after 35 days of exposure to 25 mmol/l glucose. *D*: Representative PCR showing the time required for the reversibility of the glucose effect in ROP mesangial cells. After the glucose concentration was restored to 6 mmol/l, the d(CA) repeat length changed from 24 to 22 after 7 days and back to 20 after 21 days. *E*: Representative PCR for the MMP-9 promoter region containing d(CA) repeats in 100 glomeruli isolated from four ROP mice without (ND) and with (D) streptozotocin-induced diabetes. One of the four mice with diabetes showed 24 d(CA) repeats in the MMP-9 promoter, whereas all other mice had the $d(CA)_{20}$ allele. A d(CA) repeat ladder ranging from 26 to 20 d(CA) repeats flanked by the primer sequences was used as an internal control in all gels shown.

exposure to 25 mmol/l glucose indicated the presence of mutagenesis. Uncloned mesangial cells were used to determine whether clonal selection occurred. At baseline and after 35 days of exposure to 25 mmol/l glucose, 50 single-cell clones were obtained from the mesangial cell pool. The d(CA) repeat length was determined in each mesangial cell clone. A prevalence of the $d(CA)_{24}$ allele higher than the one expected from the experiment with mesangial cell subclones described above indicated clonal selection.

Growth curves and apoptosis rate. Mesangial cells were cultured as described above, and growth curves were obtained from three independent experiments as described (22). Cells were plated in the identical manner to assess for apoptosis. Cells were collected 72 h after plating, and apoptosis was determined by a commercially available enzyme-linked immunosorbent assay kit relying on the release of histones as per the manufacturer's recommendations (Cell Death ELISA plus; Roche Applied Science, Indianapolis, IN).

Isolation of DNA, PCR analysis, and gel electrophoresis. Mesangial cells were lysed in 1× PCR buffer (Roche Applied Science) with 0.1% SDS (Sigma-Aldrich, St. Louis, MO) and 20 µg/ml proteinase K (Sigma), and PCR was performed as previously described (23).

DNA sequencing. DNA was obtained with a standard phenol-chloroform extraction after proteinase K digestion (Invitrogen, Carlsbad, CA) of 100,000 cultured cells, and differences in the d(CA) repeat length were confirmed by DNA sequencing on three randomly selected clones for each detected allele as previously described on a Sequencer 3100 from ABI (Applied Biosystems, Foster City, CA) using BigDye Terminator (23).

Induction of diabetes. Diabetes was induced in four female B6 and ROP mice at 8 weeks of age using repeated streptozocin injections as previously described (20). After 12 weeks of follow-up without insulin treatment, all mice were killed according to National Institutes of Health Animal Use and Care Guidelines, and 100 glomeruli were microdissected for PCR analysis as described above.

RT-PCR. Mesangial cells were plated into fibronectin-coated, 25-cm² flasks. The medium was changed 12 h after seeding. A cell density of 100,000 cells/cm² was consistently used. Total mRNA was extracted after 48 h by using Tri-Reagent (MRC, Cincinnati, OH), and RT-PCR was performed, as described previously (1), with cultured mesangial cells and microdissected glomeruli. The primer sequences for MMP-9 (414 bp) and glyceraldehyde-3-phosphate dehydrogenase (561 bp) were reported previously (22).

MMP-9 Activity. Mesangial cells were plated in six-well plates in medium supplemented with 20% fetal bovine serum, which was replaced with medium containing 0.1% fetal bovine serum at 12 h after seeding. Supernatants were collected after 48 h, and cell numbers were determined. Supernatants were centrifuged to remove cellular debris and were appropriately diluted with regular medium (0.1% fetal bovine serum) to normalize cell numbers (10,000 cells). Zymography was performed as described previously (1).

Microarray analysis. Microarray analysis was carried out as previously described (24–26). Two mesangial cell clones with 20 and two mesangial cell clones with 24 d(CA) repeats in the MMP-9 promoter were identified and expanded in culture medium containing 6 mmol/l glucose. Each clone had been isolated from a different mouse. All cells were harvested when they reached 70% confluence in a T75 flask (Nalge Nunc, Rochester, NY). Total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH), and RNA integrity was confirmed by agarose gel electrophoresis. Total RNA was reverse-transcribed into cDNA, followed by in vitro transcription and fluorescent labeling. Labeled cRNA was hybridized to Affymetrix gene chips (Murine Genome U74v2 Set; Affymetrix, Santa Clara, CA), and expression patterns were analyzed using the Hewlett-Packard GeneArray laser scanner (Hewlett-Packard, Palo Alto, CA).

Data analysis. Data from three independent experiments performed in vitro on mesangial cells are shown. PCR and automated sequencing were repeated twice to exclude PCR amplification artifacts. Both Spreadex and polyacryl-
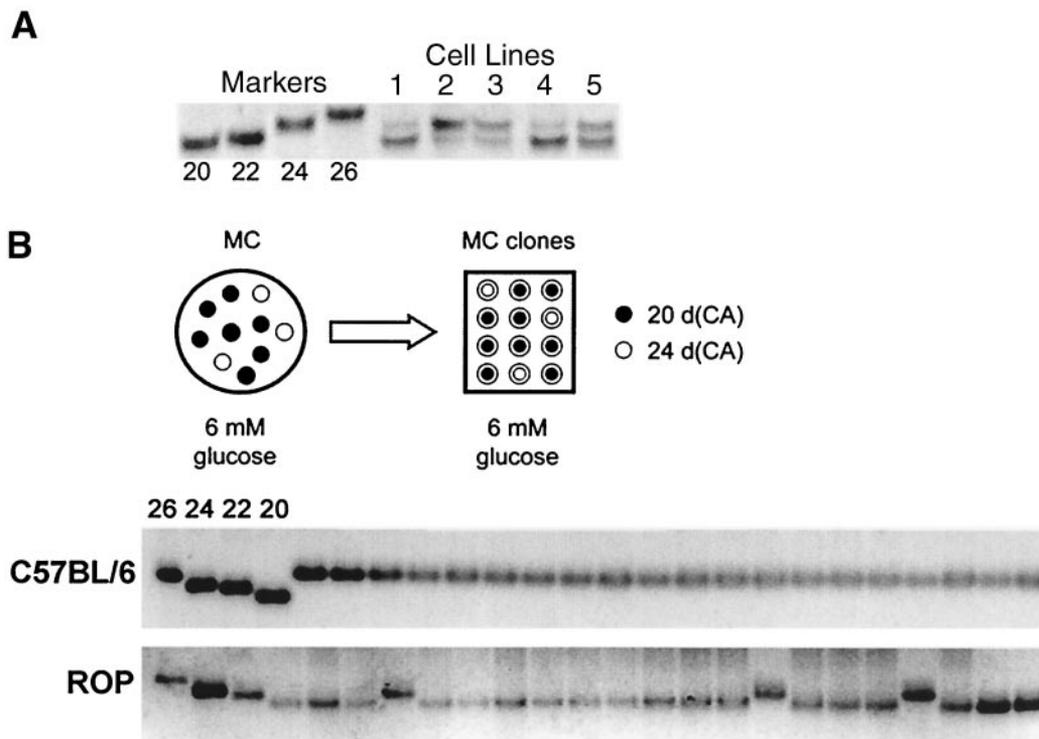
FIG. 2. ROP mesangial cell clones are heterogeneous in the length of the d(CA) repeats of the MMP-9 promoter. *A*: The d(CA) repeat length was determined by PCR in five ROP mesangial cell lines. Two alleles, $d(CA)_{20}$ and $d(CA)_{24}$, were detected in ROP mesangial cell lines. The ratio between the two alleles was variable between cell lines but reproducible within each cell line. *B*: The experimental design is shown schematically on top. One hundred single-cell clones were generated from both C57BL/6 and ROP mesangial cells. The length of the d(CA) repeat in the MMP-9 promoter was determined in all 100 clones by PCR. Representative reactions on 20 single-cell clones are shown. Mesangial cell clones from ROP mice had either 20 or 24 d(CA) repeats. All mesangial cell clones derived from C57BL/6 mice had 26 d(CA) repeats. MC, uncloned mesangial cells; MC clones, single-cell clones derived from mesangial cells. A d(CA) repeat ladder ranging from 26 to 20 d(CA) repeats flanked by the primer sequences was used as an internal control in all gels shown.

amide gels were used to confirm length polymorphisms. Microarray analysis was carried out using the software packages BRB-ArrayTools, SAM: Significance Analysis of Microarrays, and j-express (27–29). Expression data were filtered for maximum absolute signal intensity across arrays of at least 100, corresponding to the bottom 5% signal intensity, and an absolute signal difference of at least 100 between the highest and lowest signal across all arrays. All arrays were centered, followed by logarithmic transformation to the base 2. A log intensity variation filter ($P > 0.01$) was applied. Differentially expressed genes had to have at least a twofold difference. For cluster analysis, average linkage of centered genes with Euclidean distances was computed.

RESULTS

Glucose modified the d(CA) repeat length in the MMP-9 promoter of ROP mesangial cells. The length of the d(CA) repeat in the MMP-9 promoter was analyzed in mesangial cells isolated from glomerulosclerosis-prone ROP mice cultured in either 6 or 25 mmol/l glucose for 35 days. The $d(CA)_{20}$ allele was detected in ROP mesangial cells cultured in 6 mmol/l glucose, whereas the $d(CA)_{24}$ allele was found in mesangial cells exposed to 25 mmol/l glucose (Fig. 1*B*). The time required to change from one allele to another in ROP mesangial cells was studied by analyzing mesangial cell DNA collected on a weekly basis. The allelic change did not appear until 35 days (Fig. 1*C*) and was reversible when ROP mesangial cells, which had been exposed to 25 mmol/l glucose, were returned to 6 mmol/l glucose (Fig. 1*D*). We identified an intermediate allele with 22 d(CA) repeats 7 days after the glucose concentration was restored to 6 mmol/l (Fig. 1*D*). The $d(CA)_{20}$ allele became prevalent at 21 days after the glucose concentration was restored to 6 mmol/l (Fig. 1*D*).

Alterations in ambient glucose concentrations did not induce an allelic change in mesangial cells isolated from glomerulosclerosis-resistant C57BL/6 mice (Fig. 1*B*). PCR and direct sequencing were performed on DNA purified from 100 glomeruli dissected from four ROP and four B6 control mice as well as from four ROP and four B6 mice after 3 months of uncontrolled diabetes. We found that one of four ROP mice showed an increase in the number of d(CA) repeats of the MMP-9 promoter in vivo from 20 to 24 in isolated glomeruli (Fig. 1*E*). The dinucleotide repeat length did not change in glomeruli from B6 mice with diabetes (data not shown).

The d(CA) repeat of the MMP-9 promoter is polymorphic in ROP mesangial cells. Five additional mesangial cell lines from five female ROP mice were generated as described above, and the d(CA) repeat length of the MMP-9 promoter was analyzed. We detected both a $d(CA)_{20}$ and a $d(CA)_{24}$ allele with variable allele frequency in each cell line (Fig. 2*A*), giving rise to the hypothesis that a chimerism may be present. We tested this hypothesis by generating mesangial cell clones from both early and late passages (2–18) of mesangial cells isolated from ROP and C57BL/6 mice. Clones with either 20 d(CA) or 24 d(CA) repeats were found in mesangial cells from ROP mice. Among 100 clones assayed, the prevalence of clones with 24 d(CA) repeats was 13% (Fig. 2*B*). Neither time in culture nor cell density led to changes in dinucleotide repeat length in ROP mesangial cell clones (data not shown). All clones isolated from C57BL/6 mice had 26 d(CA) repeats
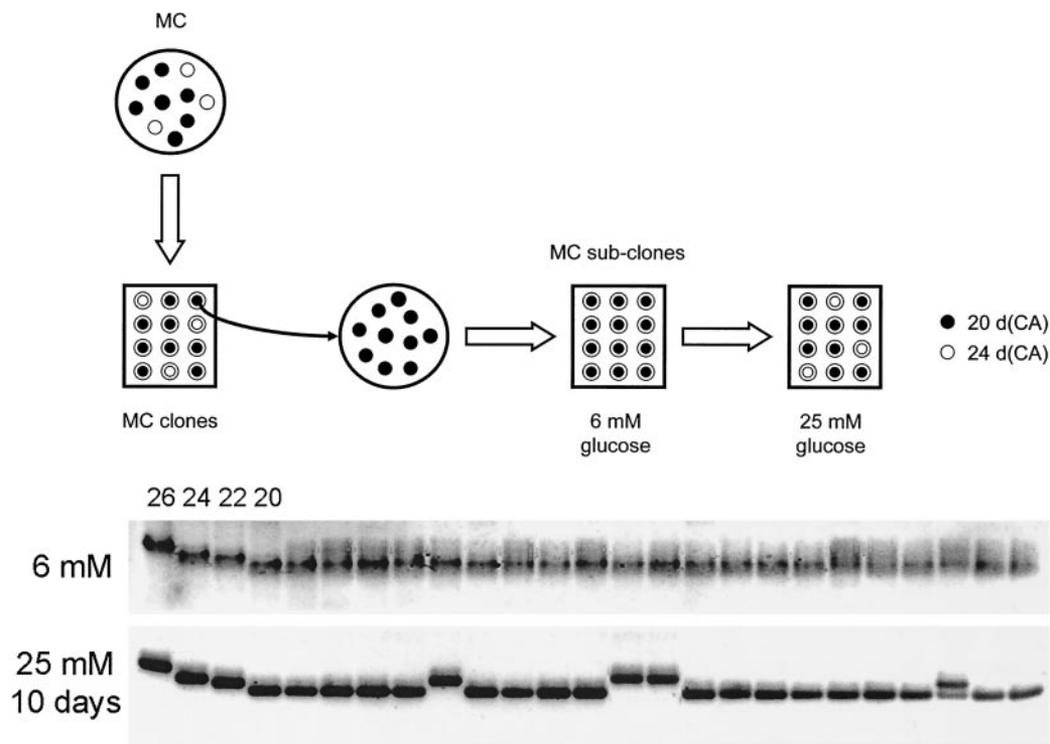
FIG. 3. Glucose (25 mmol/l) induces d(CA) insertion in the MMP-9 dinucleotide repeat. The experimental design is shown schematically on top. Single-cell clones were generated from ROP mesangial cells. One clone with 20 d(CA) repeats was selected and expanded. From this expanded mesangial cell clone, 100 mesangial cell subclones were generated. All mesangial cell subclones again had 20 d(CA) repeats. These subclones were exposed to 25 mmol/l glucose, and the d(CA) repeat length was determined by PCR in all 100 mesangial cell subclones. After 10 days of exposure to 25 mmol/l glucose, 20% of the mesangial cell subclones showed an insertion of dinucleotide repeats in the MMP-9 promoter. Shown is a representative PCR on 21 clones. Mesangial cell subclones in *lanes 5*, *10*, and *11* show d(CA) repeat expansion from 20 to 24; the subclone in *lane 19* contains both the d(CA)$_{20}$ and d(CA)$_{24}$ repeat. A d(CA) repeat ladder ranging from 26 to 20 d(CA) repeats flanked by the primer sequences was used as an internal control.

(Fig. 2*B*). Automated sequencing confirmed the results obtained by PCR (data not shown).

Glucose-induced MMP-9 d(CA) repeat expansion in ROP mesangial cell subclones. We investigated whether glucose induces reversible dinucleotide repeat expansion by studying mesangial cell subclones derived from two different mesangial cell clones with 20 d(CA) repeats. We developed 100 mesangial cell subclones from these two mesangial cell clones with 20 d(CA) repeats and exposed them to either 6 or 25 mmol/l glucose for 35 days. After 10 days of exposure to 25 mmol/l glucose, an allelic change from d(CA)$_{20}$ to d(CA)$_{24}$ was observed in 20 of the 100 mesangial cell subclones (Fig. 3). None of the mesangial cell subclones that had the d(CA)$_{20}$ allele at this time point changed to the d(CA)$_{24}$ allele during the next 25 days, so at the end of the 35-day period, the number of clones with the d(CA)$_{24}$ allele remained at 20%. None of the mesangial cell subclones cultured in 6 mmol/l glucose showed a change in the length of d(CA) repeats over this time period.

Clonal selection contributed to the change in the d(CA) repeat length in the MMP-9 promoter of ROP mesangial cell clones exposed to 25 mmol/l glucose. Exposure of uncloned mesangial cells to 25 mmol/l glucose led to a complete change of the predominant allele from d(CA)$_{20}$ to d(CA)$_{24}$ (Fig. 1*B*). However, only 20% of the mesangial cell subclones showed d(CA) repeat expansion from 20 to 24 after exposure to 25 mmol/l glucose (Fig. 3). We hypothesized that clonal selection may explain this observation. We isolated 100 mesangial cell clones from a pool of uncloned mesangial cells that had been

cultured for 35 days in 25 mmol/l glucose. We determined the d(CA) repeat length in all 100 clones and found that 100% had 24 d(CA) repeats (Fig. 4, *top*). This finding suggests that both dinucleotide repeat expansion and clonal selection contributed to the complete change in the prevalent allele, from d(CA)$_{20}$ to d(CA)$_{24}$, observed in uncloned mesangial cells after long-term exposure to 25 mmol/l glucose.

Restoration of ROP mesangial cell clones to 6 mmol/l glucose concentrations results in MMP-9 d(CA) deletions. We then restored the glucose concentration to 6 mmol/l and determined the d(CA) repeat length 10, 21, and 35 days later. We found that 100% of the mesangial cell clones returned to 20 d(CA) repeats over a period of 35 days (Fig. 4). The number of mesangial cell clones with 20 d(CA) repeats increased in a time-dependent manner and included several intermediate alleles. Thus, progressive dinucleotide deletion was responsible for the change in d(CA) repeats from 24 to 20.

ROP mesangial cells with 20 or 24 d(CA) repeats in the MMP-9 propagated in 25 mmol/l glucose have identical growth and apoptosis rates. We investigated whether mesangial cells with 24 d(CA) repeats may have a growth advantage when cultured in 25 mmol/l glucose medium. All cell lines displayed a decrease in their doubling time after exposure to 25 mmol/l glucose; however, the response was identical in lines with 20 or 24 d(CA) repeats, respectively. Data from three independent growth curves from mesangial cells with 20 and 24 d(CA) repeats in the MMP-9 promoter, which were propagated in 25
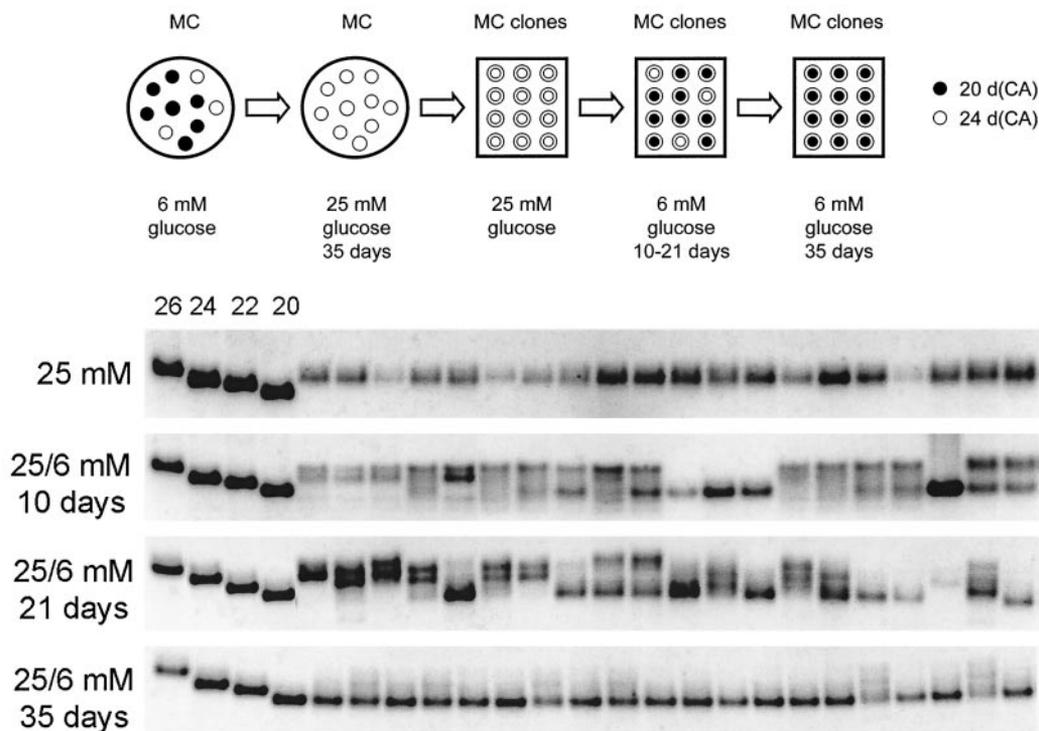
FIG. 4. Restoration of the glucose concentration to 6 mmol/l leads to deletion of d(CA) repeats in ROP mesangial cell clones. The experimental design is shown schematically on top. One hundred mesangial cell clones with 24 d(CA) repeats were isolated from mesangial cells cultured in 25 mmol/l glucose for 35 days. The d(CA) repeat length returned to 20 d(CA) repeats after restoration of the glucose concentration to 6 mmol/l in all mesangial cell clones. This occurred in a time-dependent manner. Heterozygosity and an intermediate allele with 22 d(CA) repeats were observed between 10 and 21 days after restoration of the glucose concentration to 6 mmol/l. A d(CA) repeat ladder ranging from 26 to 20 d(CA) repeats flanked by the primer sequences was used as an internal control.

mmol/l glucose, yielded identical doubling times, which were (mean [hours] $\pm$ SD) 21.0 $\pm$ 1.08 and 21.0 $\pm$ 0.52, respectively. In addition, we did not detect any difference in apoptosis rates between mesangial cells with 20 or 24 d(CA) repeats, which were (mean [percentage of positive control] $\pm$ SD) 11.3 $\pm$ 3.5 and 11.7 $\pm$ 4.5, respectively.

The d(CA)$_{24}$ allele frequency correlates with MMP-9 expression and activity. We found that the frequency of the d(CA)$_{24}$ allele in uncloned ROP mesangial cells correlates well with MMP-9 activity (Fig. 5A–C). In addition, ROP mesangial cells exposed to 25 mmol/l glucose showed a significantly higher MMP-9 expression when compared with ROP mesangial cells propagated in 6 mmol/l glucose, which decreased after ROP mesangial cells were switched back from 25 to 6 mmol/l glucose (Fig. 5D). These data are in line with previously published findings (23).

Mesangial cells with different d(CA) repeat lengths in the MMP-9 promoter are characterized by significantly different gene expression profiles. We investigated whether the difference in the d(CA) repeat length in the MMP-9 promoter is associated with differences in gene expression profiles. We compared two mesangial cell lines each with 20 and 24 d(CA) repeats in the MMP-9 promoter, respectively, using Affymetrix mouse gene chips. Of 12,488 genes in the set, 1,177 genes (9%) remained after filtering. Using SAM and cluster analysis, we identified 175 genes that showed significantly different expression levels of at least twofold between the clones with 20 and 24 d(CA) repeats, respectively, with 44 genes being higher expressed in the d(CA)$_{24}$ clones and 131 genes being higher expressed in the d(CA)$_{20}$ clones (Fig. 6). The false discov-

ery rate was computed as [median (or 90th percentile) of the number of falsely called genes] divided by [the number of genes called significant] and yielded zero for the median and 21% for the 90th percentile, respectively, for the 175 genes classified as differentially expressed.

DISCUSSION

The principal findings in this study are that ROP mice susceptible to diabetic glomerulosclerosis are characterized by the presence of mesangial cell clones heterogeneous with respect to the length of the d(CA) repeat in the MMP-9 promoter, that exposure of ROP mesangial cells to high glucose concentrations leads to d(CA) repeat expansion and clonal selection, and that returning ROP mesangial cells to an environment with normal glucose concentrations leads to d(CA) repeat deletion until the original d(CA) repeat length is reached. The finding that ROP mesangial cell clones with different d(CA) repeat length in the MMP-9 promoter have significantly different gene expression profiles adds biological relevance to these results.

We isolated mesangial cells from ROP mice and identified clones with 20 and 24 d(CA) repeats in the MMP-9 promoter, respectively, in each mouse. This is the first time such a michrochimerism of mesangial cell has been described. Microsatellite instability is one possible mechanism that could result in the presence of mesangial cell clones with different d(CA) repeat length in ROP mice. It is commonly believed that the rate of microsatellite mutations reflects both the actual rate of occurrence of muta-
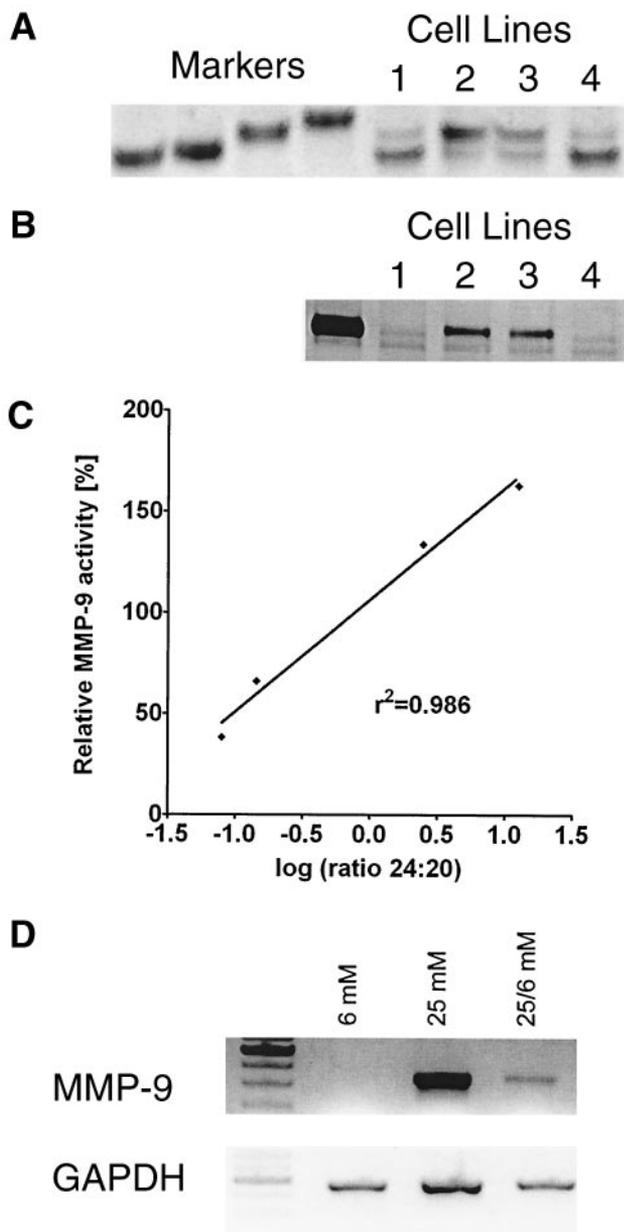
FIG. 5. Correlation of allele frequency and MMP-9 expression and activity. *A*: Representative PCR for the MMP-9 promoter region containing d(CA) repeats in uncloned ROP mesangial cells. Two alleles, $d(CA)_{20}$ and $d(CA)_{24}$, are detectable. A d(CA) repeat ladder ranging from 26 to 20 d(CA) repeats flanked by the primer sequences was used as an internal control. *B*: Representative zymography using the cell lines in *A*. Shown is the MMP-9 activity only. A positive control in the first lane confirms the identity of the bands shown. *C*: Intensities for the two alleles shown in *A* and MMP-9 activity shown in *B* were determined by densitometry. After logarithmic transformation, the ratio $d(CA)_{24}$ to $d(CA)_{20}$ was plotted against the MMP-9 activity and linear regression analysis was performed (correlation coefficient $r^2 = 0.986$). *D*: Representative RT-PCR for MMP-9 expression in ROP mesangial cells propagated in 6 mmol/l glucose, exposed to 25 mmol/l glucose for 5 weeks, and then returned to 6 mmol/l glucose medium (25/6). MMP-9 expression increases in ROP mesangial cells propagated in 25 mmol/l glucose and returns almost back to baseline after mesangial cells have been returned to 6 mmol/l glucose medium. Glyceraldehyde-3-phosphate dehydrogenase serves as a housekeeping gene.

tions and the efficiency of mismatch repair (30). This has been studied extensively in neoplastic transformation (31) and aging (32). However, we found that one of the more common enzymes involved in DNA repair, MHS2, is expressed by ROP mesangial cells, and in our experience,

ROP mice do not develop malignancies at a higher rate. In addition, we tested two randomly chosen dinucleotide repeats per chromosome (excluding X) on mesangial cell subclones derived from a clone with known d(CA) repeat length, and we could not detect any polymorphisms between the subclones (data not shown), making microsatellite instability less likely. Additional studies will be necessary to investigate the mechanisms leading to the presence of mesangial cell michrochimerism in ROP mice.

Prolonged exposure to 25 mmol/l glucose induced a change in the prevalent allele from $d(CA)_{20}$ to $d(CA)_{24}$ in mesangial cells isolated from ROP mice, and both clonal selection or dinucleotide repeat expansion may have occurred. These two possibilities were examined by studying both uncloned and cloned ROP mesangial cells. The latter were derived from a single cell with either 20 or 24 d(CA) repeats. When uncloned mesangial cells were exposed to 25 mmol/l glucose for 35 days and single-cell clones were then isolated and characterized, all of the clones had 24 d(CA) repeats. However, when ROP mesangial cell subclones that initially had 20 d(CA) repeats were exposed to 25 mmol/l glucose for 35 days, only 20% of these single-cell subclones underwent d(CA) repeat expansion from 20 to 24. These data show that although d(CA) repeat expansion occurs in ROP mesangial cells after prolonged exposure to 25 mmol/l glucose, it is not sufficient to explain why all of the cells in an mesangial cell pool exposed to 25 mmol/l glucose had the $d(CA)_{24}$ allele. Thus, a combination of dinucleotide repeat expansion and clonal selection seems to occur in ROP mesangial cells in response to elevated glucose concentrations. Clonal selection suggests a growth advantage of one clone over the other. It is interesting that we could not detect a difference in either doubling time or apoptosis rate between cell lines with 20 or 24 CA repeats, which suggests that a paracrine interaction may exist between the different clones.

Because neither passage number nor cell density influenced the d(CA) repeat length, our data indicate that glucose had mutagenic potential, which has been suggested by others (33–38). However, to our knowledge, this is the first time that a reversible d(CA) repeat expansion has been described, and the finding that dinucleotide repeat expansion occurred in 20% of cloned mesangial cells exposed to 25 mmol/l glucose is well above what is expected to occur spontaneously (39). The d(CA) repeat expansion induced by 25 mmol/l glucose in vitro was reversible in both cloned and uncloned mesangial cells when the glucose concentration was restored to 6 mmol/l. Thus, progressive d(CA) deletion, not clonal selection, seemed to be the underlying mechanism for this finding. The presence of the intermediate allele $d(CA)_{22}$ at early time points after glucose concentrations were restored to normal suggested that dinucleotide repeats were progressively deleted. This observation provides strong evidence that two different pathways are responsible for the induction and the reversibility of glucose-induced changes in vitro, d(CA) repeat expansion and clonal selection in the former and dinucleotide deletion in the latter. It is interesting that two lines of mesangial cells isolated from ROP mice after 12 weeks of uncontrolled streptozotocin-induced diabetes all showed the $d(CA)_{24}$ allele, irrespective of the glucose concentration of the cell culture media
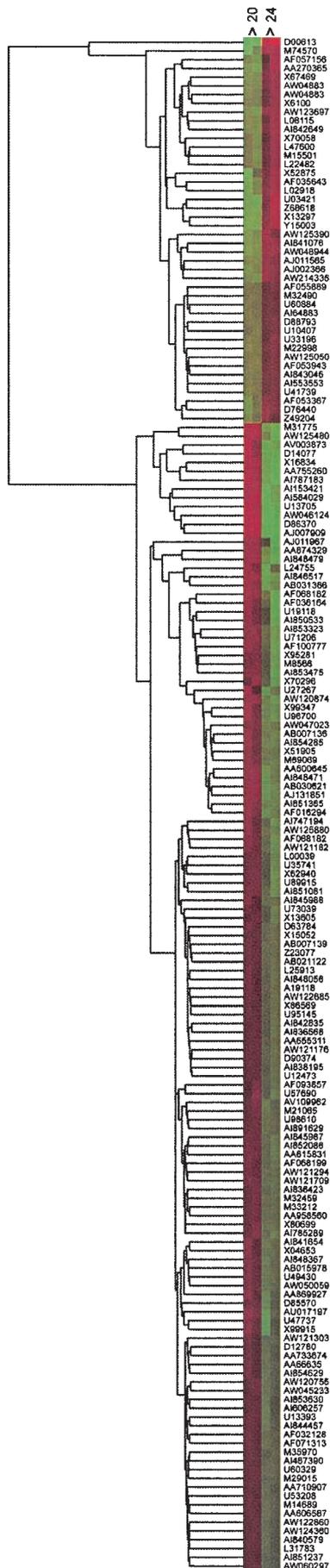
used (data not shown). This suggests an important difference between the findings of effect of glucose in vitro and of diabetes in vivo, and studies are ongoing to explore this observation.

Michrochimerisms may be biologically relevant if they are associated with differences in gene expression or cell behavior. We have previously shown that the d(CA) repeat region of the MMP-9 promoter influences gene expression in mesangial cells (23), and the results in this study confirm that a correlation exists between MMP-9 activity and $d(CA)_{24}$ allele frequency. In addition, we could show that after exposure to 25 mmol/l glucose, the MMP-9 mRNA expression increases in conjunction with the switch in genotype from $d(CA)_{20}$ to $d(CA)_{24}$. This may have direct implications on the pathogenesis of diabetic nephropathy, because an increase in MMP-9 expression has been reported to characterize, if not precede, early diabetic nephropathy (40–43). However, this is not without controversy because a decreased expression of MMP-9 has been shown to be associated with diabetic nephropathy by others (44). Thus, we investigated whether additional genes are differentially expressed in cell lines with 20 and 24 d(CA) repeats, respectively. We performed gene expression profiling on four independent cell lines, two of which had the $d(CA)_{20}$ and two of which had the $d(CA)_{24}$ allele. A subset of genes showed a highly significant difference between the two groups, and gene expression levels differed by as much as 40-fold. We analyzed two arrays in each group, with each array representing an independent cell line. In this setting, a stringent statistical analysis would likely yield a large type 2 error, whereas with a less stringent analysis, the type 1 error would be unacceptable. This is reflected by the computed false discovery rate. For this reason, we decided not to speculate about the association of individual genes with the pathogenesis of diabetic nephropathy. However, our data show that the gene expression profiles of cell lines with 20 and 24 d(CA) repeats in the MMP-9 promoter are significantly different, underscoring the potential biological relevance of the observed michrochimerism.

We showed that a change of the predominant allele of the MMP-9 promoter also occurred in vivo after 3 months of uncontrolled diabetes, which adds biological relevance to our in vitro findings. The observation that only one of four diabetic mice showed d(CA) repeat expansion may be explained by the fact that mesangial cell turnover is much slower in vivo than it is in vitro, where mesangial cells typically exhibit a doubling time of ~24 h, and that DNA replication may be necessary for this change in d(CA)

FIG. 6. Gene expression profiling of ROP mesangial cell clones. Gene expression profiling was performed on two cell lines derived from mesangial cell clones with either 20 or 24 d(CA) repeats in the MMP-9 promoter using Affymetrix mouse gene chips (Murine Genome U74v2 Set). Of 12,488 genes in the set, 175 showed significantly different expression levels of at least twofold between the clones with 20 and 24 d(CA) repeats, respectively. Of those, 44 genes showed higher expression in the $d(CA)_{24}$ clones and 131 genes showed higher expression in the $d(CA)_{20}$ clones. Shown is the dendrogram computed for average linkage and centered correlation using Euclidean distances and the expression profiles for 175 differentially expressed genes. Genes that are underexpressed in one group are depicted in green, and overexpressed genes are depicted in red; brightness correlates with absolute expression levels. The gene bank accession number for each gene is shown on the right. 20, mesangial cell clone with the $d(CA)_{20}$ repeat; 24, mesangial cell clone with the $d(CA)_{24}$ repeat.

repeat length to occur. Thus, a longer duration of diabetes may be required, and additional markers for clonal selection in vivo may have to be identified.

In summary, we demonstrate that ROP mice are characterized by the presence of mesangial cell clones heterogeneous with respect to the length of the d(CA) repeat in the MMP-9 promoter. Exposure of ROP mesangial cells to high glucose concentrations in vitro leads to clonal selection and reversible d(CA) repeat expansion. Mesangial cell clones with different d(CA) repeat lengths showed a significantly different gene expression profile. We conclude that mesangial cell michrochimerism may be a marker for the susceptibility to glomerulosclerosis, that dinucleotide repeat expansion may be a novel mechanism for glucose-induced changes in gene expression, and that dinucleotide repeat expansion and/or clonal selection may partially explain the change in mesangial cell phenotype in individuals with diabetes.

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