Somatic mutations are frequent and increase with age in human kidney epithelial cells

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We have used a primary cloning assay to determine the frequency of 6-thioguanine (TG)-resistant tubular epithelial cells in kidney tissue from 72 human donors ranging in age from 2 to 94 years. The frequency of TG-resistant mutants ranged from $\sim 5 \times 10^{-5}$ for donors in the first decade of life to $\sim 2.5 \times 10^{-4}$ for donors in the eighth and later decades of life. Two different statistical analyses indicated that this increase in mutant frequency is exponential with age. We also observed a 2-fold higher TG-resistant mutant frequency in nephrectomy kidneys containing a coincident renal carcinoma. DNA sequence analyses revealed HPRT gene mutations in each of 14 TG-resistant mutants from seven unrelated donors. Thirteen of these 14 mutants resulted from independent mutational events. These results suggest that somatic mutations are common in renal—and perhaps in other human—epithelia, and thus could play an important role in the genesis of age-associated disease.

INTRODUCTION

Somatic mutations were first suggested, nearly 40 years ago, to play a role in the pathogenesis of age-associated disease (1,2). However, comparatively few data exist as yet on somatic mutation frequency as a function of age, or on the role of specific somatic mutations in the genesis of age-associated disease apart from cancer. Particularly surprising is the lack of data on somatic mutations in epithelial cells. Epithelial cells play critical roles in tissue structure and function, and are the target tissue for development of most human age-associated neoplasms (3).

We have used the *HPRT* locus to quantify and characterize somatic mutations occurring *in vivo* in human kidney cortical epithelial cells. The mutational target in these analyses, the X-linked *HPRT* locus, encodes the purine salvage enzyme hypoxanthine phosphoribosyl transferase (EC 2.4.2.8). The *HPRT* locus is widely used for mammalian mutation analyses due to a combination of facile somatic cell and molecular genetic methods, and dispensability of the HPRT protein in most somatic cells (4–6).

The frequency of renal cortical cells resistant to the purine analogue 6-thioguanine (TG) was quantified using a primary epithelial cell cloning assay. Mutations were characterized by DNA sequence analysis of *HPRT* cDNA or genomic DNA segments recovered from independent, TG-resistant mutants. These data indicate that *HPRT*-deficient kidney tubular epithelial cells are common, arise from a wide range of mutational events, and show an exponential increase in frequency over the human lifespan. These results, to the best of our knowledge, represent the first analysis of the frequency and molecular basis for somatic mutation in a human epithelial cell lineage as a function of donor age.

RESULTS

Human kidneys from three sources were analyzed: unused transplant kidneys, nephrectomy specimens and autopsy kidneys. Virtually all (~95–98%) of the primary cell clones we grew from collagenase-dissociated kidney cortex displayed an epithelioid morphology. There was considerable heterogeneity in the proliferative capacity of both primary TG-resistant and control clones, with only a subset (5–10%, depending on the donor) reaching sufficient size (~10^6 cells or 20 cumulative population doublings) for initial molecular genetic analysis.

Immunocytochemical analyses of several independent TG-resistant and control clones from different donors confirmed their epithelial character: cytokeratin 8 and stratum corneum-associated cytokeratin expression were observed in an intermediate filament pattern with variable vimentin co-expression (Fig. 1A–C), whereas no desmin, muscle actin or factor VIII expression was observed (Fig. 1D). The remaining few percent of colonies we recovered had a fibroblastoid morphology and fibroblast-associated immunocytochemical markers (data not shown).

We were able to grow epithelioid colonies from 66 of 72 (or 92% of) donor kidneys. A plot of the TG-resistant mutant frequency as a function of donor age for 61 of these kidneys is shown in Figure 2. Clones from five additional kidneys were used to establish mutation analysis methods. Clones could not be grown from the remaining six kidneys due to microbial contamination (three of the nephrectomy, and one of the transplant, kidneys) or, in the case of two autopsy kidneys, unexplained low colony forming efficiencies (CFEs). Transplant and nephrectomy specimens had comparable CFEs (mean $228\,730 \pm 23\,997$ S.E. colonies/g tissue vs. $212\,210 \pm 21\,810$ S.E. colonies/g tissue, respectively) that were four-fold higher than
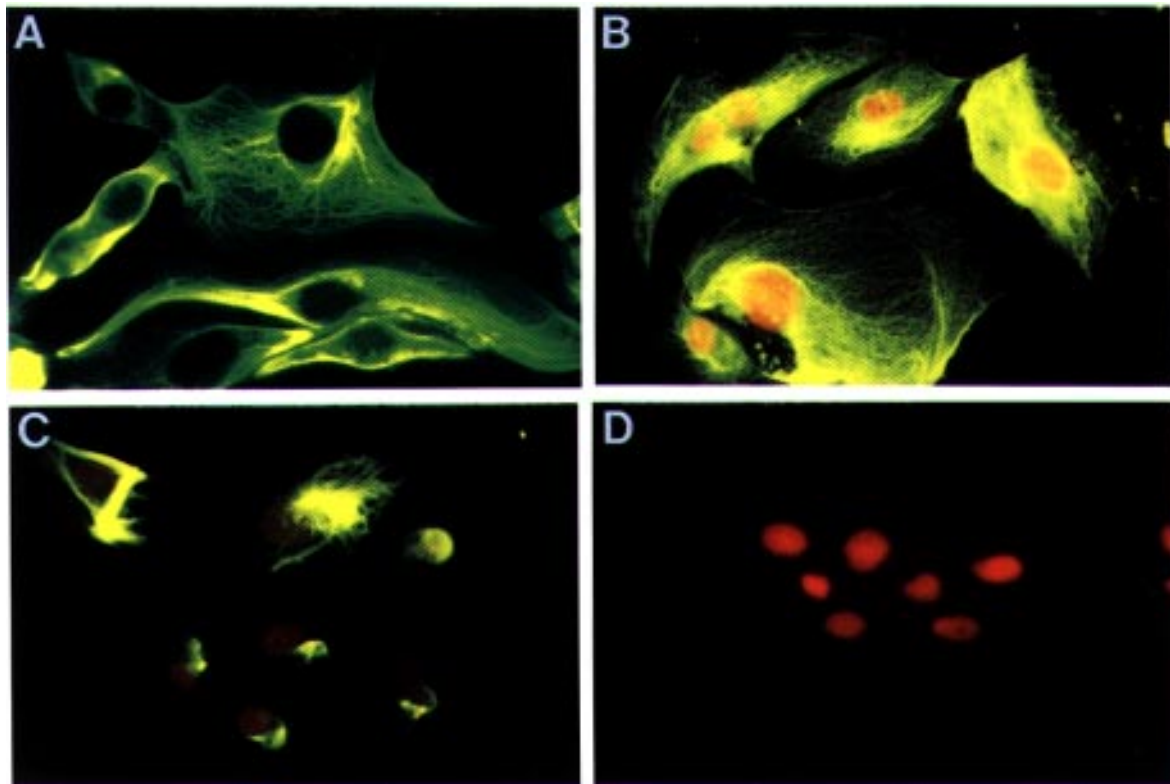
Figure 1. Immunocytochemical characterization of TG-resistant primary cell clones from human renal cortex. A panel of monoclonal antibodies to intermediate filament and cell type-specific markers were used for indirect immunofluorescence analyses. Strong staining in an intermediate filament distribution pattern was observed with antibodies to cytokeratin 8 (**A**) and stratum corneum-associated cytokeratins (**B**). Less intense staining with a more variable pattern was observed with antibodies to vimentin (**C**). (**D**) shows a lack of indirect immunofluorescence staining with antibodies to muscle actins. A nuclear DAPI counterstain has been included in (B–D).

the CFE observed in three autopsy kidneys (mean $56\,583 \pm 12\,472$ S.E. colonies/g tissue). CFEs were calculated per gram of dissociated tissue as it was difficult to determine, by morphology alone, the absolute number and proportion of viable and dead cells in collagenase-generated cell suspensions. The post-mortem intervals for the three autopsies included here ranged from 2 to 6 hours. We have also found that viable epithelial cells can, in selected instances, be grown from human autopsy kidneys >18 h after death (data not shown).

Two different statistical analyses were performed on the frequency data shown in Figure 2, least squares regression diagnostics and the Box-Cox lambda power transformation. Both analyses indicate that mutant frequency increases exponentially with age. Least squares regression diagnostics supported an exponential relationship between TG-resistant mutant frequency and age: *p*-values for the age coefficient are both significant, but favor the exponential model ($p = 0.000002$, versus $p = 0.0003$ for a linear model). More importantly, in these analyses an exponential model explained a substantially larger portion of the variability in mutant frequency data ($R^2 = 0.32$ versus $R^2 = 0.20$). Comparable results were obtained when these analyses were repeated using subsets of the data sorted by kidney source. There does not appear to be an influence of gender on the age regression, although the sample size for females was small ($n = 13$). A log transformation of TG-resistant frequency versus age was suggested by the Box-Cox lambda statistic ($\lambda = -0.04$, 95% confidence intervals $= -0.34, 0.24$), where the null hypothesis of

a linear increase in TG-resistant frequency with age could be strongly rejected ($\chi^2 = 44.9$, $p < 0.0001$). The age slope for this increase, estimated by fitting a regression line to log-transformed data, was $0.0095 \log$ (TG-resistant mutant frequency) y^{-1}, or ~1% per year of age.

Mutant frequency analyses also indicated that there were higher mutant frequencies in kidneys from patients undergoing nephrectomy for a primary renal carcinoma. Adding a variable to analyses that identified kidneys containing a coincident renal carcinoma substantially improved the fit of an exponential model relating mutant frequency to donor age ($t = 2.869$, $p = 0.0057$). When donors aged 40 to 70 were examined to minimize the lack of overlap in the age ranges of kidney donors with or lacking a coincident renal carcinoma, we again found higher mutant frequencies in tumor-containing kidneys (mean TG-resistant mutant frequencies of 2.4×10^{-4} with, versus 1.3×10^{-4} without, a coincident renal carcinoma; $t = 2.17$, $p = 0.036$).

This mutant frequency difference is unlikely to reflect the presence of tumor in the tissue sampled: histopathologic examination of hematoxylin and eosin-stained tissue sections from a subset of donor kidneys ($n = 25$) that included kidneys with coincident renal carcinomas revealed mild or moderate, age-associated changes though no evidence of tumor (data not shown). This frequency difference is also unlikely to represent region-specific or sampling differences, as comparable mutant frequencies were observed in kidneys where two or four independent tissue samples from a kidney were processed in
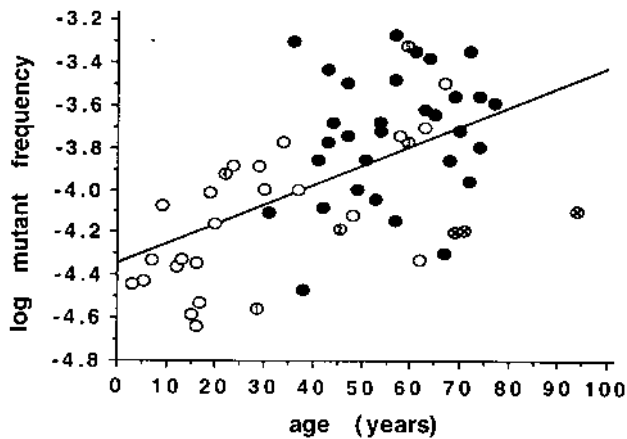
Figure 2. Frequency of TG-resistant primary renal cell clones as a function of age and donor kidney source. The log frequency of TG-resistant mutant clones in kidney tissue from 61 different human donors is shown as a function of donor age. Each circle represents one of the 61 donors, where the source of the kidney (unused transplant, nephrectomy or autopsy kidney) and additional donor data are given below. Least squares regression analysis was used to fit and to determine the slope of the mutant frequency versus age regression line. ($\bigcirc$), unused transplant kidney; ($\bullet$), nephrectomy kidney with coincident renal carcinoma; ($\otimes$), autopsy kidney. Open circles with numbers indicate nephrectomy kidneys that contain the indicated coincident disease process: ①, coincident ischemic tubular necrosis; ②, horseshoe kidney; ③, coincident hematoma; ④, hypoplastic kidney; and ⑤, coincident transitional cell carcinoma.

parallel. In one of these analyses, an unused transplant kidney from a 19 year old male donor had mutant frequencies of 9.5 and 10.1×10^{-5} in samples taken from opposite poles of the kidney. The other analysis, of a transplant kidney from a 9 year old male donor, yielded mutant frequencies of $7.1–10.1 \times 10^{-5}$ in samples taken from the anterior and posterior aspect of each pole.

A summary of data from DNA sequence analysis of *HPRT* cDNA or genomic DNA fragments from 14 TG-resistant mutants from seven unrelated donors is shown in Table 1. All 14 TG resistant mutants contained an identifiable *HPRT* mutation. Eight of the 14 mutations represent small changes (transition or transversion mutations, a 4 bp deletion and a 1 bp insertion) in the *HPRT* coding region. The remaining six mutations included two characterized and four putative splice junction mutations, three of which lead to the inclusion of intron 1 sequence between exons 1 and 2 of the mature *HPRT* mRNA (Table 1). Eight of these mutations appear to be novel, as they are not included in the most recent release of the human *HPRT* mutation database (7) nor, to the best of our knowledge, have they been previously reported. These novel mutations include two of the six single base substitutions (mutants 55-003 and 57-124, Table 1), the tandem base substitution (54-026), the single base insertion (53-015), the 4 bp deletion (56-016), and three of the putative splice junction mutations (55-011 and 55-012, and 57-207). Thirteen of the 14 mutations detailed in Table 1 appear to have arisen as independent mutational events. The exception, a putative splice junction mutation associated with a 65 bp insertion in two of three TG-resistant mutants (from donor HK55, Table 1), most likely arose from a single mutational event.

Table 1. HPRT mutations in TG-resistant clones

*All donors listed are males. The letter in parentheses after each donor indicates the source of kidney tissue used: t, unused transplant kidney; s, surgical nephrectomy specimen.

†Nucleotide numbering indicates the position(s) of the indicated change(s) in the human *HPRT* genomic DNA sequence (39).

‡The mutational consequences of putative splice junction mutations were determined by cDNA sequence analyses, while the consequences of base substitution, deletion and insertion mutations were inferred from the human *HPRT* cDNA and protein sequences (42,43).

DISCUSSION

The quantitative and molecular data described above provide an interesting glimpse of somatic mutation in a human epithelial cell lineage. Morphologic and immunocytochemical features of TG-resistant and control clones grown from human kidney cortex suggests that a majority ($\geq 95\%$) were epithelial-derived. The most probable origin for these clones is from convoluted tubule epithelial cells, as these are the most abundant of the cortical

epithelial cell types (8,9). It should be possible to more precisely identify the specific tubular region(s) from which these cells are derived by further studies that use a combination of immunocyto-chemical, *in situ* hybridization or PCR and functional analyses (9).

Renal tubular epithelial cells are an interesting target tissue for somatic mutation analyses. They are metabolically active and consume large amounts of oxygen; they perform a majority of the secretory and resorptive work needed to process glomerular filtrate; they are mitotically competent, though largely quiescent, in the absence of a growth stimulus or injury; and they are the cell type from which the predominant primary human renal tumor, renal adenocarcinoma, arises (8,10–13). The ability to obtain proliferating colonies of renal tubular epithelial cells from routine human autopsy kidney is also of interest as it expands the investigative potential of the autopsy (see, e.g., 14).

Virtually all of the TG-resistant mutants we recovered are likely to have arisen *in vivo*. We applied TG selection within 24 hours of plating cell suspensions, and grew presumptive TG-resistant mutants continuously in TG until mutation analysis. A delay or phenotypic 'lag' is commonly seen in the expression of induced *HPRT* mutations, and low levels of *HPRT* enzyme activity (<2% of control activity) have been found in most human and animal cells resistant to 10 µg/ml (60 µM) TG (4–6,15–17). These observations suggest that continuous TG selection should select strongly for the growth of preexisting *HPRT*-deficient mutants that arose *in vivo*, and against new TG-resistant mutants that arose during cell isolation or rare, TG-sensitive cells that can survive, though not grow, in the presence of TG (18 and unpublished results).

Our initial molecular genetic analyses of TG-resistant and control clones focused on the 5–10% of clones from each donor that had sufficient proliferative potential to grow to ~10^6 cells. The considerable heterogeneity we observed in the proliferative capacity of both TG resistant and control clones is most likely the result of clonal attenuation, in which proliferating primary somatic cells continually segregate progeny with variable, though declining, growth potential (19). All of the TG-resistant clones we examined contained *HPRT* mutations that would eliminate or severely attenuate *HPRT* enzyme activity (4–7), and virtually all appear to have arisen as independent mutational events (Table 1). In order to determine a representative spectrum of *HPRT* mutations occurring in human kidney tubular epithelial cells, we are characterizing a larger number of mutations in clones with a range of different growth potentials.

The TG-resistant mutant frequencies we observed in primary renal epithelial cells is approximately 10-fold higher than have been described in human peripheral blood T lymphocytes (20). One explanation for this could be differences in the TG concentration at which the mutant frequency reaches a plateau. This possibility is unlikely for at least two reasons. The mutant frequency for kidney tubular epithelial cells falls to a minimum at 60 µM TG, and mutants recovered at this TG concentration contain *HPRT* mutations similar to those recovered from human T-lymphocytes or continuous cell lines at comparable TG concentrations (7,20,21). The higher mutant frequency in renal epithelial cells is also unlikely to be explained by an age-depend-ent decrease in CFE, as has been described for T-cells (20); the CFE of renal epithelial cells does not appear to decline with age.

One unexpected finding in mutant frequency analyses was a consistently higher frequency of TG-resistant mutants in donor kidneys containing a coincident renal carcinoma. This increase cannot be explained simply by the greater average age of nephrectomy versus other kidney donors. An elevated mutant frequency in tumor nephrectomy kidneys could have several different and mechanistically interesting explanations: patients with renal carcinomas could have had unusual or excessive exogenous mutagen exposure; carcinoma patients could have a heritable or acquired DNA repair deficit or genomic instability; or they could have relaxed somatic selection against, and thus a lower probability of eliminating, TG-resistant *HPRT*-deficient mutant cells as they arise. Additional data on the spectrum of *HPRT* mutations recovered from nephrectomy kidneys may begin to shed mechanistic light on these intriguing possibilities. The observation of a higher mutant frequency in kidneys having a coincident renal carcinoma may be clinically relevant, as there is renewed interest in nephron sparing therapy in which a renal carcinoma, though not adjacent normal kidney, is removed at the time of surgery (21).

Our data indicate that TG-resistant renal cortical epithelial cells are frequent, and that the frequency of mutant cells appears to increase exponentially with donor age. This apparent exponential increase, and comparable age-dependent increases in TG-resistant mutant frequencies in human and mouse T-lymphocytes (20,22–25), are compatible with 'error catastrophe' and other somatic mutation-based theories of aging (26). One as yet unexplained finding is that we have seen comparable age-dependent increases in TG-resistant mutant frequency in human and dog kidney, though not in mouse kidney or mouse skeletal muscle fibroblasts (18,27). This difference is unlikely to reflect strong, species-specific selection against HPRT-deficient cells: the fre-quency of TG-resistant T-lymphocytes increases in aged mice in tissue where the selection against HPRT-deficient variants appears to be more stringent than in slowly dividing tissues such as kidney (25,28,29).

These results provide, to the best of our knowledge, the first analysis of mutant frequency and of the molecular characteristics of somatic mutations arising in a human epithelial cell lineage. Somatic mutations in kidney, and perhaps in other human epithelia, are common and appear to increase exponentially with age. The presence of independent mutational events in different TG-resistant mutants from individual kidneys indicates that a simple enumeration of TG-resistant colonies can, in most instances, be used to estimate *in vivo* mutation frequencies. It should also be possible to recover and analyze large numbers of independent mutations from single donor kidneys. This will allow us to better determine the spectrum, or frequency and distribution of different molecular types, of *HPRT* mutations occurring in kidney epithelial cells *in vivo*. These data will also allow us to determine whether the kidney *HPRT* mutation spectrum differs in important ways from *HPRT* mutational spectra observed in T lymphocytes or other human cells (7,20). These approaches should allow us to develop a better understanding of the role of somatic mutation accumulation and of specific types of mutations in the loss of renal function and elevated risk of malignancy that accompany human aging (26,30–34).

MATERIALS AND METHODS

Cell culture

A total of 72 human kidneys, from three sources, were used in this study: (i) kidneys obtained for renal transplantation (*n* = 27) by

the Northwest Kidney Center (Seattle, WA) that could not be used for technical reasons; (ii) nephrectomy specimens ($n = 40$) from the University of Washington and Swedish Hospital Medical Centers (Seattle, WA). A majority (36 of 40) consisted of normal tissue from kidneys removed for histopathologically verified renal adenocarcinoma or, in one case, invasive non-papillary, poorly differentiated transitional cell carcinoma. None of these renal carcinoma patients had received radiation or chemotherapy prior to surgery. The remaining four nephrectomy specimens came from horseshoe or hypoplastic kidneys (one each), or from nephrectomy kidneys containing hematoma or ischemia-associated tubular necrosis; and (iii) autopsy kidneys ($n = 5$) from the rapid autopsy program of the University of Washington Alzheimer's Disease Research Center. All five autopsy kidneys were from patients with pathologically confirmed Alzheimer's disease. Transplant kidneys were perfused with Belzer's solution (Dupont) supplemented with 200 000 units/l penicillin G sodium, 4 mg/l dexamethasone and 0.4 ml/l insulin, after which vessels were clamped and the kidneys placed on ice for transportation. The range of times on ice for the specimens used for these studies was 24 to 96 h. Nephrectomy and autopsy specimens were aseptically dissected, and tissue samples transported in cell culture medium (DMEM; Dulbecco-Vogt modified Eagle's medium supplemented with 100 units/ml of penicillin G sodium and 100 µg/ml of streptomycin) supplemented with 30 mM HEPES buffer (pH 7.4). Grossly normal cortical tissue from the pole opposite tumor was used from nephrectomy specimens containing a carcinoma. Tissue samples from a subset of the kidneys ($n = 25$) were paraffin embedded, sectioned and stained with hematoxylin and eosin for morphologic analysis. Independent tissue samples from opposite poles or from the anterior and posterior cortex at each pole were obtained from a subset of kidneys to examine intra-organ variability in mutant frequencies.

Primary clones were grown from cell suspensions prepared by collagenase dissociation of kidney cortical tissue. Cortical tissue samples (~3 g/kidney) were aseptically dissected to remove renal capsule and medullary tissue, then minced to obtain ~1 mm^3 tissue fragments. These were washed 2–3 times in Ca^{+2} and Mg^{+2}-free phosphate-buffered normal saline (pH 7.1) (PBS; Grand Island/BRL), then digested with type 1 collagenase (Sigma) to generate cell suspensions. Washed tissue fragments were resuspended in 50 ml of a 1:1 (v/v) mixture of type 1 collagenase (1 mg/ml in PBS) and DMEM. Digestion was performed in a 100 ml homogenizing flask with gentle stirring at 37°C. Supernatant was collected after 1 h and remaining tissue was digested with an additional 50 ml of the collagenase:DMEM mixture for 1 h at 37°C. Supernatants were combined and centrifuged at $2600 \times g$ for 5 min. The resulting cell pellet was resuspended in 50 ml of growth medium (DMEM supplemented with 16% (v/v) fetal bovine serum) for plating.

The CFE of cell suspensions was determined by inoculating 0.1 ml of a 1:100 dilution of cell suspensions into 15 ml of growth medium in each of twenty 100 mm culture plates. Thioguanine (TG)-resistant clones were isolated by inoculating 0.5 ml of undiluted cell suspension in 10 ml of growth medium into each of an additional eighty 100 mm plates. After 24 h, these plates were refed with 15 ml of growth medium supplemented with 10 µg/ml (60 µM) 6-thioguanine (TG). Plates under TG selection contained $5.2–10.6 \times 10^4$ viable cells/plate. All plates were grown in a water vapor-saturated 37°C, 7% CO_2 incubator, and were refed with fresh growth medium twice each week. TG-resistant colonies were grown continuously in the presence of TG-containing medium. Colonies on 10 CFE and at least 20 TG selection plates were counted after staining plates with 0.5% (w/v) crystal violet in 20% (v/v) ethanol. Mutant frequencies were calculated by dividing the total number of TG-resistant colonies on selection plates by the mean number of colonies on CFE plates corrected to take into account dilution differences at the time of inoculation. TG resistant and control clones were isolated for each kidney using 10 mm glass cloning cylinders. We isolated multiple TG-resistant clones from a randomly chosen subset of donors from whom we received large amounts of kidney tissue. These mutants were analyzed to determine whether different TG-resistant mutants contained independent mutational events. TG-resistant and control clones for these initial mutation analyses were grown to $5–10 \times 10^6$ cells/clone, then PBS washed, pelleted and stored at –80°C for molecular analyses.

Immunocytochemical analyses

Early passage cells, from colonies that had undergone 6 to 8 population doublings, were plated in growth medium on multiwell teflon-coated slides (Meloy Laboratories, Springfield, VA) and allowed to attach overnight, then PBS washed and fixed in 95% ethanol or acetone (–20°C) for 10 min before being air-dried. Indirect immunofluorescence was performed as previously described using monoclonal antibodies to vimentin (43βE8); cytokeratin 8 (35βH11); stratum corneum keratins (34βE12), muscle actins (HHF35); and commercially available monoclonal antibodies to desmin and factor VIII (35,36).

Mutation analyses

DNA and RNA for mutation analyses were obtained from TG-resistant and control clones using a modification of the method developed by Henikoff that omitted the DNase step (37). Aliquots of the final preparation were resuspended in TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) for RT-PCR or PCR amplification as previously described (38). The primers used for cDNA amplification were 1676 (–) and 41502 (+) (see primer listing below). The resulting product was a 710 bp DNA containing the complete 657 bp human *HPRT* protein coding region. Genomic DNA amplifications were performed with primers flanking the intron/exon boundaries of *HPRT* exon 8 [39848 (–) and 40195 (+)]. The resulting 387 bp DNA fragment contained both the 3′ acceptor splice site of intron 7 and the 5′ donor splice site of intron 8. Direct DNA sequencing of amplified fragments was used to identify *HPRT* mutations. DNA fragments were purified by electrophoresis through 1% low melting point agarose gels, excised and melted at 70°C for 10 min. Aliquots were then added directly to sequencing reactions. Sequencing was performed using the Cycle Sequencing System kit and primers end-labelled with γ^{32}P-ATP and polynucleotide kinase according to the manufacturer's protocol (Life Technologies). Each strand was sequenced with four different primers. Control clones isolated from the same donor were amplified, sequenced and run in parallel. Putative mutations were verified by repeating amplification(s) and DNA sequencing in all instances. The primers used for amplification and sequence analyses are listed below. Primer position refers to the position of the 3′ end of the primer in the human *HPRT* genomic DNA sequence (39). Primers followed by a '(+)' hybridize to the *HPRT* mRNA or to the sense strand of genomic DNA, while primers followed by a

'(–)' hybridize to the mRNA complementary strand of *HPRT* cDNAs or to the antisense strand of genomic DNA.

Position	Sequence
1676(–)	5′-CCGCGCGCTGGCCGGATCCGTT-3′
14873(–)	5′-GGGTGTTTATTCCTCATGGAC-3′
16639(+)	5′-CACAGAGGGCTACAATGTGATGGCCTCCC-3′
16760(–)	5′-GTGATGGATCCATTCCTATGACTGTAG-3′
27891(+)	5′-TTAGGTACCCTGTTGACTGGTCATT-3′
34946(+)	5′-CAAAGCTTGCATTGTTTTGCCAGTGTC-3′
35016(–)	5′-CAGTAGGATCCAAAGATGGTCAAGGTCGC-3′
39848(–)	5′-GACCCCACGAAGTGTTGGA-3′
40195(–)	5′-GGTGCTGGAAGGAGAAAACA-3′
41502(+)	5′-GTTTCCAAACTCAACTTGAATTCTCATC-3′

Statistical analyses

Two different statistical analyses, least squares regression analysis and the Box-Cox λ power transformation, were performed to investigate the relationship of mutant frequency to age. For least squares regression analysis, linear and exponential models of mutant frequency as a function of age were fit and compared using *p* values and R^2 percent of variance explained. The influence of kidney source on mutant frequency was tested using regression analysis of the log-transformed data after controlling for donor age. Mutant frequency data were also analyzed using the Box-Cox λ power transformation (40) as implemented by Venables and Ripley (41). The Box-Cox λ statistic estimates, by maximum likelihood, the power transformation of mutant frequency as a function of age that best fits the data. A Box-Cox λ of 0 indicates a log transformation and exponential relationship between mutant frequency and donor age, while a λ of 1 indicates no transformation and a linear relationship between mutant frequency and age. If 95% confidence intervals around the estimated λ (λ̂) do not contain 1, the null hypothesis of a linear relationship can be rejected in favor of a greater than linear increase in mutant frequency with age. The increasing variability seen in mutant frequency with age may itself favor the log transformation in Box-Cox λ analyses. Thus it is important to confirm the results of Box-Cox λ analyses with a second independent method such as least squares regression analysis.

ACKNOWLEDGMENTS

ABBREVIATIONS

CFE, colony forming efficiency; DAPI, 4′,6′-diamidino-2-phenyl-indole; DMEM, Dulbecco-Vogt modified Eagle's minimal essential medium supplemented with 100 units/ml penicillin G sodium and 100 μg/ml of streptomycin sulfate; *HPRT*, hypoxanthine phosphoribosyltransferase gene; PBS, phosphate-buffered normal saline, pH 7.1; S.E., standard error, TG, 6-thioguanine.

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