Kidney International, Vol. 40 (1991), pp. 906–912

Stable cell line of T-SV40 immortalized human glomerular visceral epithelial cells

FRANÇOISE DELARUE, ANGELA VIRONE, JACQUELINE HAGEGE, ROGER LACAVE, MARIE-NËLLE PERALDI, COLETTE ADIDA, ERIC RONDEAU, JEAN FEUNTEUN, and JEAN-DANIEL SRAER

INSERM U 64, Hôpital Tenon, Paris, and CNRS UA 1158, Institut Gustave Roussy, Villejuif, France

Stable cell line of T-SV40 immortalized human glomerular visceral epithelial cells. Human subcultures (third passage) of glomerular visceral epithelial cells (VEC) isolated from one month old kidney were successfully transfected by two recombinant plasmids containing the cloned oncogenes from the simian virus 40 large T antigen and H-ras gene. One postcrisis cell clone (56/10 A1) was selected, propagated and characterized. One hundred percent of the 56/10 A1 cells (current passage >100th; doubling time 30 hrs) expressed the nuclear T-SV40 antigen assayed by IF; the cells failed to express H-ras (RNA blot analysis). Immortalized cells were morphologically and phenotypically compared to parental cell type (third passage). Phenotypic characterization of the 56/10 A1 cells was achieved using indirect immunofluorescence (IF) and immunogold silver staining coupled to bright field and epipolarization microscopy. Both parental and 56/10 A1 cells displayed positivity for cytokeratin, CALLA and PHM5, whereas von Willebrand factor was not detected in the two cell types. Since we have previously shown that human glomerular epithelial cells in culture synthetize plasminogen activator (PA) related compounds, we investigated the secretion pattern of these products in parental and transfected cells. Zymographic analysis of secreted PA related compounds revealed production of free urokinase (u-PA) and type 1 plasminogen activator inhibitor (PAI-1) complexed to tissular plasminogen activator (t-PA). Finally, in the transfected cells, increased cGMP generation under atrial natriuretic factor (ANF) stimulation agreed with previous work performed on nontransfected human VEC. In conclusion, the establishment of a human permanent cell line which retains most of the phenotypic features of parental glomerular visceral epithelial cells should represent a new tool to study human glomerular cell functions.

Glomerular cells from several mammalian species have been used to study biochemical and physiological properties of the glomerulus [1–3]. Nevertheless, the use of such cell preparations is restricted by two major obstacles: their limited life span in vitro and the frequent contamination of the cultured cells by an undesired cell type when working on uncloned cell lineages. At the present time, one method to abolish these problems is the establishment of cell lines from cloned cells immortalized and/or transformed by recombinant cDNA procedures or viral infection. Transgenic mice for the early region of the DNA tumor virus simian SV40 (T-SV40) have been used to clone and maintain glomerular epithelial, mesangial and endothelial cell cultures in long-term follow-up [4]. The authors report that the cells maintain several characteristics of their normal counterparts. Concerning human cells close to viral infection, the only useful cDNA recombinant procedure is DNA transfection using transforming and/or immortalizing oncogenes. Furthermore, oncogene-mediated induction of differentiation of rat granulosa cells has been reported after double transfection with T-SV40 and H-ras oncogene [5]. At the present time there is no cloned differentiated human glomerular cell culture of a prolonged lifespan.

In our work, we have isolated and cloned VEC transfected with T-SV40 and H-ras from a one-month-old human baby kidney. The cell cultures have been propagated over 100 times and maintain several predominant characteristics of normal human visceral epithelial cells (VEC).

Methods

Glomerular visceral epithelial cells preparation and transfection

VEC were cultured from a normal human baby kidney (one month old) judged to be unsuitable for transplantation. Cells were obtained, as previously described, by collagenase digestion of isolated glomeruli [6]. The isolated cells were then suspended in RPMI supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin.

The DNA transfection was performed on the third subculture. The cells were transfected by the calcium phosphate procedure [7]. They were plated at $5 \cdot 10^5$ before the transfection and were exposed to a coprecipitate of calcium phosphate containing 5 μg of PAS plasmid and 5 μg of activated human PEJ ras plasmid. PAS expressed the early region of SV40 under the control of its own regulatory region deleted of its replication origin. It was cloned in pBR 322 [8]. PEJ ras was cloned at the Bam H1 site of pBR 322. While and after the transfection procedure, cells were seeded in defined medium: DMEM: Ham's/F12 1:1 (vol/vol), 10 mM HEPES, 2 mM glutamine, 5 μg/ml insulin, $5 \cdot 10^{-8}$ M dexamethasone, $3 \cdot 10^{-8}$ M sodium selenate, 5 μg/ml transferrin, 100 U/ml streptomycin, and 100 U/ml penicillin, supplemented with 1% FCS.

Received for publication March 25, 1991
and in revised form June 3, 1991
Accepted for publication June 7, 1991

After four weeks in culture, some colonies were isolated by the cloning ring method, the cells were resuspended and seeded in 35 mm petri dishes. After one week, the new colonies of VEC were individually picked up in the cloning ring; cells were resuspended in culture medium, counted and diluted to seed them in 96 well plates in order to obtain a mean final concentration of one cell per well. By this procedure, five clones were grown in five wells containing initially one cell. They were explanted separately and one of them (56/10 A1) was selected to be propagated. All the subcultures had been cryopreserved and were easily subcultured after thawing. Growth rates of the 56/10 A1 cells were determined at passage 5 (P) 53 and 100.

Morphological studies

Morphological studies were done between P45 and P55 for transfected cells and at P3 for the parental type cells. For the bright field microscopy examination, cells were cultured in plastic petri dishes, then fixed for 30 minutes in PBS 1.5% glutaraldehyde and stained by Giemsa solution. For the scanning electron microscopy (SEM) examination, the cells were cultured on multiwell glass slides, fixed in PBS 2.5% glutaraldehyde and dehydrated in acetone. They were treated at the critical point (Polaron E 3000 apparatus) by carbon streams, and then observed by SEM Geol at 7 kV.

Identification of SV40 T antigen (Tag), PHM5, CALLA and cytokeratin antigens

Parental cells from the same kidney and transfected cells were treated at the same passages. For the identification of Tag, cells were fixed in acetone/methanol (70/30) at −20°C for ten minutes, then kept at −20°C and treated with the anti-Tag (mAb) for one hour at room temperature. Tag was then revealed by biotinylated anti-mouse IgG antibody (15 μg/ml) and fluorescein streptavidin (FITC) 10 μg/ml.

For PHM5, a monoclonal antibody specific for glomerular epithelial cells [9, 10], cells were fixed in 4% paraformaldehyde for 10 minutes and then incubated with an anti-PHM 5 mAb (Australian Monoclonal Development) and submitted to a similar subsequent procedure as the cells treated for Tag examination.

For cytokeratin antigen identification, a marker of epithelial differentiation, the cells were permeabilized with 0.05% saponin for five minutes, fixed in 1.5% glutaraldehyde PBS for ten minutes, rinsed and incubated first for one hour with a 10% monoclonal anti-cytokeratin (kidney-associated simple epithelia; Amersham, UK) in PBS-BSA, rinsed, and subsequently incubated for 30 minutes in biotynilated anti-mouse IgG (15 μg/ml). For examination by epipolarization the biotin was visualized by incubation in 0.5% streptavidin, gold labeled (Sigma) in PBS-BSA. The cell preparation was then washed and observed after Silver amplification (Janssen kit). CALLA, which is also expressed by human visceral epithelial cells [11], was identified according to this procedure using a mAb (IOT5 Immunotechnology) as first antibody, but after 4% paraformaldehyde fixation of the cells. In each case, nonspecific staining was determined by omitting the first antibody.

Finally, the cells were also treated with an anti-factor VIII related antigen mAb (Dako Patts, Denmark). Preparations were examined and photographed with a Leitz microscope.

Determination of cellular cGMP generation by atrial natriuretic factor

cGMP was measured in transfected cells between P70 and P80 at cell confluency in 25 mm petri dishes using a kit from the Radiochemical Center (Amersham) as previously described [12]. cGMP determination was performed after five minutes of incubation either with rat atrial natriuretic factor (ANF) (1.28) from Calbiochem (San Diego, California, USA) at 10^{-7} M, sodium azide (N_3Na) at 10^{-3} M or at steady state. Results are expressed as mean ± SE as fmol/ml of conditioned medium.

Fibrinolytic related compounds analysis

Subconfluent cells were incubated in multidish wells in serum and hormonal-free defined medium for 24 to 48 hours as described above. Conditioned culture medium was then removed and used for zymographic analysis to detect plasminogen activators as described [13]. The plasminogen activator inhibitor 1 (PAI-1) was detected by reverse fibrin autography as previously described [14].

Results

Morphology and growth rate

Figure 1 shows the morphological features observed both in third subculture of nonstransfected VEC and at P50 for 56/10 A1 cells. Figure 1 a and b show photomicrographs of the two cell types which grow forming a continuous layer. As shown the two cell types exhibited an epithelial cell morphology with a homogenous aspect of regular, cobblestone-like polygonal cells. Nevertheless, as compared to parental cell type, 56/10 A1 cells displayed some special morphological features. They were smaller, with leaky junctions and larger intercellular spaces.

SEM studies (Fig. 1 c and d) also show some differences between parental type and 56/10 A1 cells, the latter being smaller and having a cellular body less spread. Parental type cells seemed more attached to the substratum than 56/10 A1 cells. In addition, they exhibited large and thin protrusions and some possessed microvilli. As suspected, on phase contrast microscopy examination, the intercellular junctions were tighter between the parental type cells than between transfected cells. Finally, but rarely, 56/10 A1 cells exhibited aspects of multilayer growing cells.

Concerning the growth rate, the growing properties of A56/10 A1 cells displayed characteristics of immortal epithelial cell lines, since at the time of this study they had been propagated for 19 months and passaged over 100 times. Their population doubling time was 30 hours during the logarithmic phase of growth at P53 and P100. As compared, the parental cell type subcultures were lost after P5.

This growth phenotype of transfected immortalized cells was undoubtedly due to the expression of the integrated DNA of PAS plasmid and the subsequent T-SV40 gene expression. As far as ras was concerned, Southern blotting of Bam H1 digested transfected cells' DNA, probed with the labelled purified ras cDNA, exhibited positive hybridization with a signal estimated to correspond to 3 to 5 integrated copies. Nevertheless, Northern blotting of total cellular RNA probed with the same labelled, purified RAS DNA failed to detect any hybridization (data not shown).

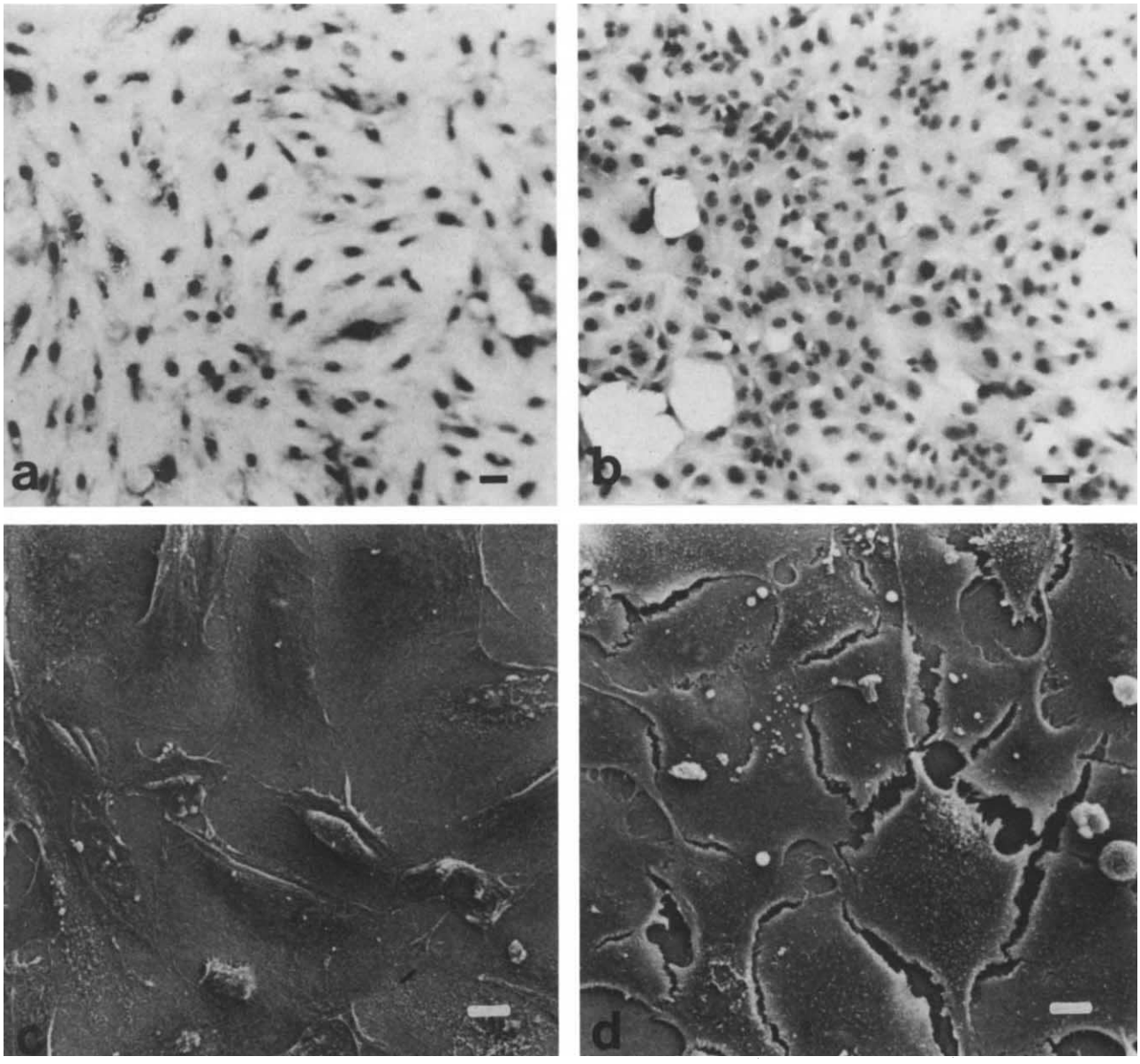

Fig. 1. *Parental nontransfected human VEC at P3 (a) and T-SV40 transfected cloned 56/10 A1 cells at P45 (b).* The photographs show the general aspect of the cultures; bar = 10 μm. Cells morphology is more detailed by SEM studies for parental cells (c) and 56/10 A1 cells (d); bar = 5 μm.

Therefore, we conclude that although pEJ RAS plasmid is integrated in genomic DNA, the oncogene is not transcripted. The immortalization of 56/10 A1 cells seemed, therefore, due to the T-SV40 as confirmed by immunocytochemical studies.

Immunocytochemical studies

As shown in Figure 2, Tag can be easily identified by indirect immunofluorescence in 56/10 A1 cell nuclei (100% of positive staining at P20 and P50). Three differentiation markers of VEC were subsequently investigated, both for the parental cells and 56/10 A1 cells using specific antibodies. Two antibodies were directed against cell surface antigens of VEC: PHM5 [9, 10] and CALLA [9]. As shown by indirect immunofluorescence, both parental cells and 56/10 cells were homogeneously stained by these antibodies, although 56/10 A1 cells seemed to have a less pronounced staining than parental cells (Fig. 3 a and b). PHM5 has been shown to recognize a carbohydrate moiety of a podocyte surface protein which is contained in the glycocalyx of human visceral glomerular epithelial cells in vivo [10]. CALLA has been shown to be expressed in the brush border of proximal tubular cells and in the membrane podocytes in the human kidney in vivo [11]. Immunogold silver staining with anti-CALLA antibody and epipolarization microscopy allowed an identification of a strictly restricted membranous distribution of CALLA in the two cell lines (Fig. 3 c and d). Finally, with the same procedure we identified the presence of cytokeratin, a marker of epithelial differentiation and one of the cytoskeletal proteins of VEC in culture [2]. This antigen was associated with

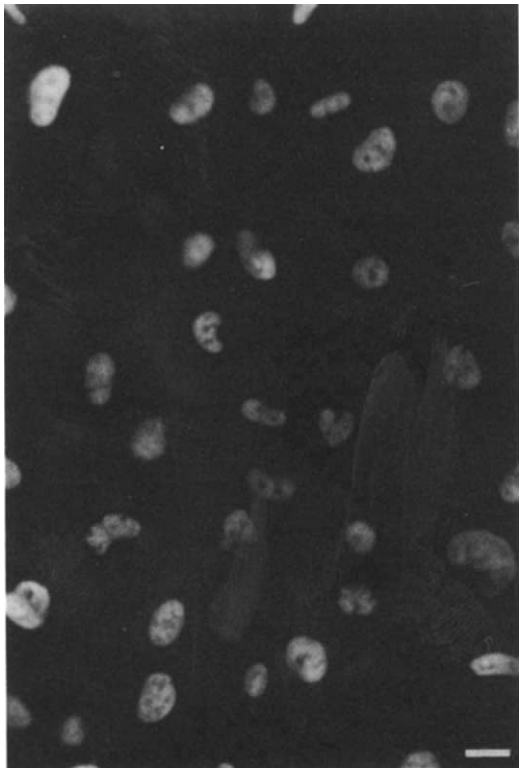

Fig. 2. *Indirect immunofluorescence staining of the intranuclear antigen encoded by T antigen SV40 in 56/10 A1 cells at P50.* All the nuclei are positively stained; bar = 10 μm.

the cytoplasm in the two cell lines (Fig. 3 e and f, Fig. 4a). No evident pattern of filaments was observed. As shown in Figure 4b, nonspecific staining by the sensitive method IGSS was very low. Factor VIII-related antigen was not detectable, which permitted the distinction between these glomerular epithelial cells and endothelial cells.

cGMP generation

56/10 A1 cells were sensitive to N_3Na and ANF as previously demonstrated in nontransfected human glomerular VEC [12]. N_3Na (10^{-3} M) stimulated the cGMP generation about three times more than nonstimulated cells. Linear ANF (1-28) at 10^{-7} M markedly stimulated cGMP generation of about 400 times (Basal conditions: 9.8 ± 2.5; N_3Na: 32.2 ± 5.1; 10^{-7} M ANF: 3970 ± 350 fmol/well; mean ± SEM of 2 separate experiments made in duplicate).

Zymographic analysis of fibrinolytic related compounds

Little is known on specific compounds secreted in vitro by VEC. We have previously demonstrated that subcultures of human VEC can synthesize and release fibrinolytic related compounds [15]. So, we looked for the ability of the 56/10 A1 cell line to release fibrinolytic related compounds. As demonstrated in Figure 5a, zymographic analysis of the conditioned medium revealed a main plasminogen activator released by 56/10 A1 cells with an apparent molecular weight of 53 kD, comigrating with purified u-PA, and which can be neutralized by anti-u-PA antibody. The high molecular weight form of PA corresponded to a PAI-1-t-PA complex as confirmed by immunoneutralization with specific antibodies. Free PAI-1 was also demonstrated by reverse fibrin autography (Fig. 5b). Thus, concerning the pattern of synthesis of plasminogen activators and their inhibitor PAI-1, 56/10 A1 cells behaved as nontransfected human cultured VEC [15].

Discussion

In this study we report the successful transfection and transformation by the viral oncogene, T-SV40 of cloned human VEC, isolated from normal human glomeruli. The initial population contained VEC, which are the most abundant cells at the early phase of the culture, but initial contamination by endothelial cells which do not grow in the standard conditions, and some possible mesangial cells. It is now well established that the integration of T-SV40 DNA in the genome of mammalian epithelial cells can induce cell transformation of secondary cultures [5, 16, 17]. Transformed cells display a high rate of in vitro proliferation, as do rabbit proximal tubular cells infected with the entire viral particle [17] and glomerular visceral epithelial cells isolated from mice transgenic for T-SV40 [4]. In the two latter studies the authors reported a doubling time of thirty hours, which is the same as for the 56/10 A1 cells in this study. In our work, the 100% positivity of nuclear staining for T-SV40 antigen clearly established that the transformed phenotype of 56/10 A1 cells was due to T-SV40, since, in counterpart H-ras failed to be transcripted in spite of its integration in the host genome. Although transcription of H-ras was not detectable, one cannot exclude that its integration plays a role in the differentiation of the transfected cells, as has been previously reported for granulosa cells [5].

The homogeneous characters of the 56/10 A1 cells can be clearly demonstrated considering both the cloning procedure and the immunocytochemical and physiological studies. Indeed, initially several clones were derived for each one from a single cell. We chose the 56/10 A1 clone because it was the one which best retained VEC characteristics. Morphologically 56/10 A1 cells display some features of transformed cells; although they show a cobblestone-like appearance similar to parental cells, they tend to be smaller and to grow more densely. They grow with enlarged intercellular spaces as compared to parental cells and they exhibit thin and large protrusions. The majority of these features have been also reported by MacKay et al for the SV40 transgenic mice glomerular visceral epithelial cell line [4]. Importantly, the immunological characteristics of the transformed cells agree fully with those of parental cells, attesting first that they are of VEC type, and second that they keep the parental phenotype over successive passages. Both CALLA and PHM5 antigens are membranous antigens of glomerular VEC [9–11], as cytokeratin is one of the compounds of their cytoskeleton in culture [2]. Although we used a sensitive IGSS method we did not find an evident pattern of cytokeratin filaments in these cells. This may be related to the small amount of cytoskeletal protein in podocytes in vivo [2]. Our cultured cells do not exhibit factor VIII-related antigen, thus allowing their distinction from endothelial cells. Another well established characteristic of VEC is their ability to synthetize fibrinolytic related compounds according to the same pattern that of non-transformed human VEC, with a predominant secretion of

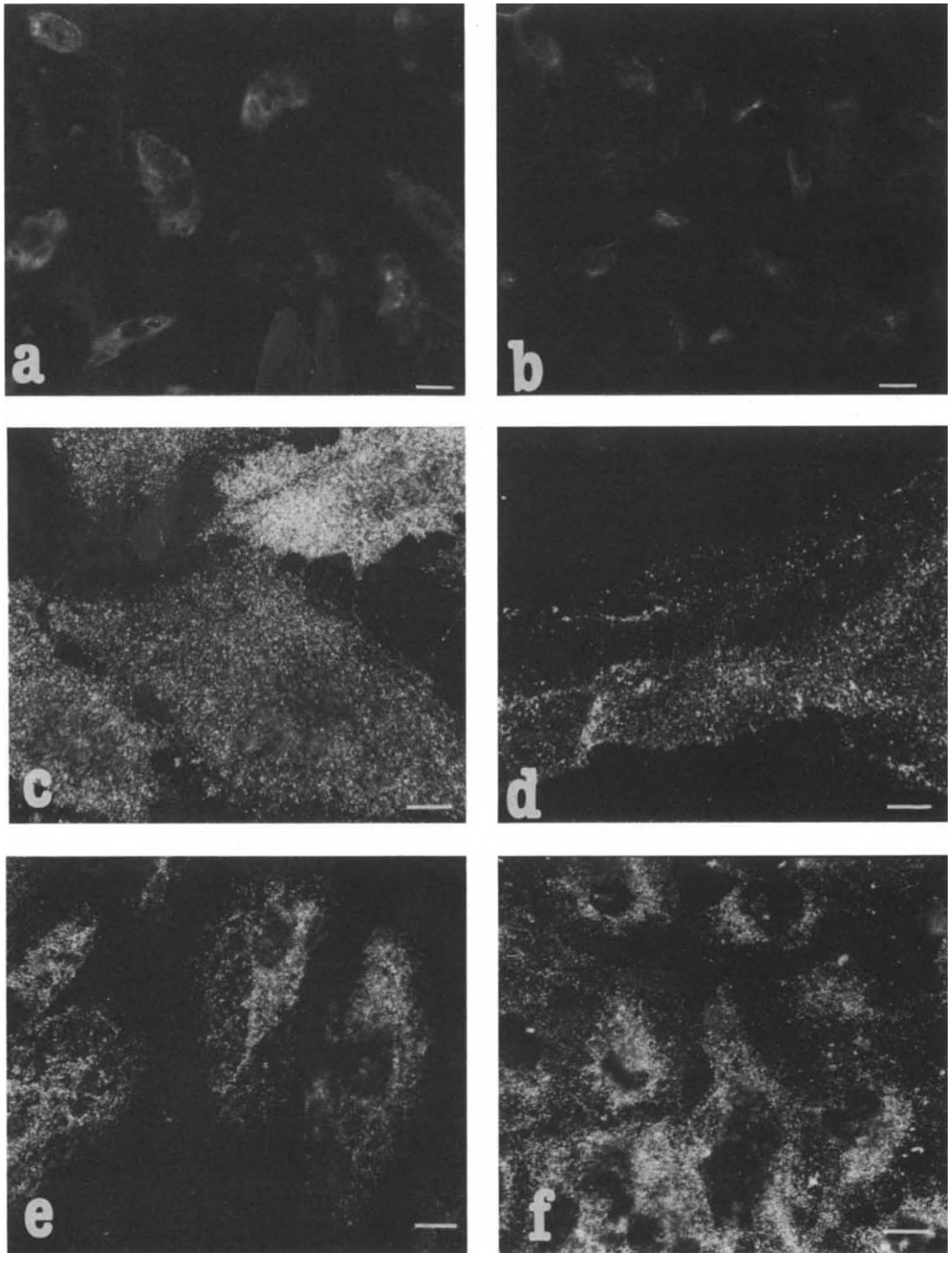

Fig. 3. *Identification of PHM5, CALLA and cytokeratin antigens in parental nontransfected VEC (left panel) and T-SV40 transfected 56/10 A1 cells (right panel).* The membranous distribution of PHM5 (a, b) (bar = 10 μm) and CALLA (c, d) antigen are observed. In contrast, a cytoplasmic location of the cytokeratin antigen (e, f) for the two cell lines (bar = 5 μm) has to be noted.

free u-PA and to a lesser extent of a PAI-1-t-PA complex [15]. This pattern of plasminogen activator release is quite specific for these cells, and different from that of human cultured mesangial cells which predominantly release free PAI-1 and PAI-1-t-PA complex [18]. Finally, as previously shown for human VEC [12], the cGMP generation by 56/10 A1 cells can be

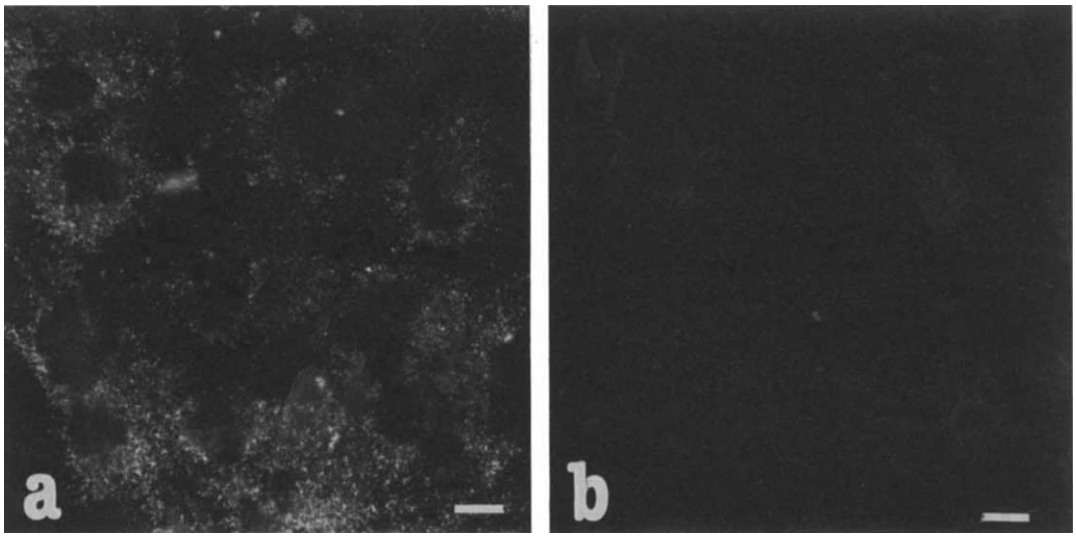

Fig. 4. *(a) Immunogold silver staining (IGSS) of cytokeratin antigen in T-SV40 transfected 56/10 A1 cells observed by epipolarization; (b) control, same incubation method as in (a) but primary anticytokeratin antibody was omitted (bar = 5 μm).*

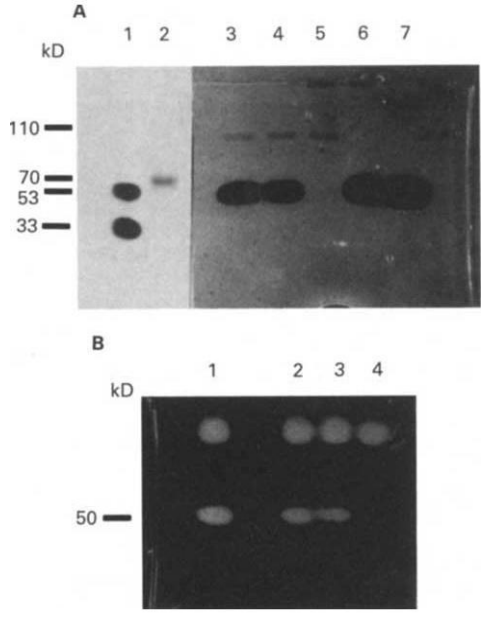

Fig. 5. A. *Zymographic analysis of 56/10 A1 cells conditioned culture medium.* Standard human u-PA and t-PA are shown on lanes 1 and 2, respectively. The conditioned medium contains mainly a 53 kD form of u-PA (lane 3) as well as high molecular weight form of 110 to 120 kD. Immunoneutralization with specific antibodies allows to determine the antigenic type of PA. The preincubation with anti-u-PA IgG (5 μg/ml) inhibits the 53 kD form (lane 5), whereas both anti-t-PA IgG (5 μg/ml) and anti-PAI-1 antiserum (1/50 dilution) inhibited the higher molecular weight form (lanes 6 and 7 respectively). Lane 4 is conditioned culture medium mixed with nonspecific goat IgG (10 μg/ml). **B.** Reverse fibrin autography of conditioned culture medium. Lane 1, purified human PAI-1; lanes 2, 3, 4, conditioned culture medium alone or after preincubation with nonspecific IgG or anti-PAI-1 antiserum, respectively. Note the presence of a 160 kD area of inhibition in all samples due to presence of anti-u-PA IgG needed to reveal the 50 kD free PAI-1.

markedly increased by linear ANF (1-28) stimulation, suggesting that they also possess ANF receptors linked to guanylate cyclase.

Importantly, the kidney used for transfection of glomerular cells came from a one-month-old baby. We used it to improve the potential rate of proliferation and differentiation. It has to be noted that both parental and transfected cells from this kidney exhibit very similar characteristics of glomerular epithelial cells from adult kidneys, as has been previously reported [15]. Since then, we performed cotransfections of glomerular cells from older kidney donors (6 months to 5 years and 50 years old) which were also successful (data not shown).

In summary, we conclude from the present findings that T-SV40 oncogene can induce immortalization of human VEC. This has allowed to us to establish a cloned line of human VEC which, despite some morphological features of transformed cells, retains numerous specific determinants of normal VEC. Thus, 56/10 A1 cells may provide a powerful tool to study human glomerular visceral epithelial cell biology.

Reprint requests to Françoise Delarue, INSERM U64, Hôpital Tenon, 4 rue de la Chine, 75020 Paris, France.

References

1. Kreisberg JI, Karnovsky MJ: Glomerular cell in culture. *Kidney Int* 22:439–447, 1983
2. Striker GE, Striker LJ: Biology of disease—glomerular cell culture. *Lab Invest* 53:122–129, 1985
3. Kreisberg JI, Hassid A: Functional properties of glomerular cells in culture. *Miner Electrol Metab* 12:25–31, 1986
4. MacKay K, Striker LJ, Elliot S, Pinkert CA, Brinster RL, Striker GE: Glomerular epithelial, mesangial and endothelial cell lines from transgenic mice. *Kidney Int* 33:677–684, 1988
5. Amsterdam A, Zauberman A, Meir G, Pinhasi-Kimhi O, Shu BS, Orem M: Cotransfection of granulocells with simian virus 40 and Ha-RAS oncogene generates stable lines capable of induced steroidogenesis. *Proc Natl Acad Sci USA* 85:7582–7586, 1988
6. Striker GE, Killen PD, Farin FM: Human glomerular cells in vitro: Isolation and characterization. *Transplant Proc* 12:88–100, 1980
7. Wigler H, Pellices A, Silverstein S, Axel R: DNA mediated transfer of the adenine phosphorinosyltransferase locus into mammalian cells. *Cell* 14:725–731, 1978
8. Benoist C, Chambon P: In vivo sequence requirements of the SV40 early promoter region. *Nature* 290:304–309, 1981

9. HANCOCK WW, ATKINS RC: Monoclonal antibodies to human glomerular cells: A marker for glomerular epithelial cells. *Nephron* 33:83–90, 1983
10. KERJASCHKI D, POCZEWSKI H, DEKAN G, HORVAT R, BALZAR E, KRAFT N, ATKINS RC: Identification of a major sialoprotein in the glycocalyx of human visceral glomerular epithelial cells. *J Clin Invest* 78:1142–1149, 1986
11. BOUCHER A, DROZ D, ADAFER E, NOEL LH: Relationship between the integrity of Bowman's capsule and the composition of cellular crescents in human crescentic glomerulonephritis. *Lab Invest* 56: 526–533, 1987
12. ARDAILLOU N, NIVEZ MP, ARDAILLOU R: Stimulation of cyclic GMP synthesis in human cultured glomerular cells by atrial natriuretic peptide. *FEBS Lett* 204:177–182, 1986
13. LOSKUTOFF DJ, VAN MOURIK JA, ERICKSON LA, LAWRENCE D: Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proc Natl Acad Sci USA* 80:2956–2960, 1983
14. VAN MOURIK JA, LAWRENCE DA, LOSKUTOFF DJ: Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by bovine endothelial cells. *J Biol Chem* 259:14914–14921, 1984
15. RONDEAU E, OCHI S, LACAVE R, HE CJ, MEDCALF R, DELARUE F, SRAER JD: Urokinase synthesis and binding by glomerular epithelial cells in culture. *Kidney Int* 36:593–600, 1989
16. EMAMI S, MIR L, GESPACH C, ROSSELIN G: Transfection of fetal rat intestinal epithelial cells by viral oncogenes: Establishment and characterization of the E1A-immortalized SLC-11 cell line. *Proc Natl Acad Sci USA* 86:3194–3198, 1989
17. VANDEWALLE A, LELONGT B, GENITEAU-LEGENDRE M, BAUDOUIN B, ANTOINE M, ESTRADE S, CHATELET F, VERROUST P, CASSINGENA R, RONCO P: Maintenance of proximal and distal cell functions in SV40-transformed tubular cell lines derived from rabbit kidney cortex. *J Cell Physiol* 141:203–221, 1989
18. LACAVE R, RONDEAU E, OCHI S, DELARUE F, SCHLEUNING WD, SRAER JD: Characterization of a plasminogen activator and its inhibitor in human mesangial cells. *Kidney Int* 35:806–811, 1989