Localization of Tamm–Horsfall-glycoprotein-like immunoreactivity in cultured baby-hamster kidney cells, shown by immunofluorescence and by light- and electron-microscopic immunoperoxidase techniques

Krishan L. SIKRI,*|| Charles L. FOSTER† and R. Derek MARSHALL‡§

Departments of *Anatomy and †Physiology, St. Mary's Hospital Medical School, London W2 1PG, and

‡Division of Biochemistry, University of Strathclyde, Glasgow G4 0NR, Scotland, U.K.

(Received 7 August 1984/Accepted 4 October 1984)

Tamm–Horsfall glycoprotein was isolated from hamster urine, and antiserum against it was produced in rabbits. IgG was isolated from the antiserum. Immunocytochemical methods were used to localize Tamm–Horsfall-like immunoreactivity in three substrains of baby-hamster kidney (BHK) cells. Indirect immunofluorescence techniques showed that, in two substrains (BHK-21/C13/2P and BHK-21/C13/3P), a proportion of the cells fluoresced brilliantly, whereas those of the third substrain (BHK-21/ICRF) were totally negative. Related findings were obtained by the immunoperoxidase optical-microscopic technique. From the results of immunoperoxidase techniques using the electron microscope, it was concluded that the substance was present in association with the plasma membranes of the reacting cells. Our data suggest that the line of baby-hamster kidney cells, BHK-21/C13, may contain cells of renal-tubular epithelial origin, and that the proportion of these may be variable from one subculture to another.

Dunstan *et al.* (1974) observed that sonicated preparations of an established line of baby-hamster kidney cells, BHK-21/C13/2P (MacPherson & Stoker, 1962; Capstick *et al.*, 1966), contained material that was immunologically cross-reactive with antisera raised against human, rabbit and hamster urinary Tamm–Horsfall glycoprotein (Tamm & Horsfall, 1950, 1952). The presence of the glycoprotein in a major proportion of a sample of the cultured kidney cells was later demonstrated by an indirect immunoperoxidase technique, and it was found that the action of crystalline trypsin, under conditions that did not change the permeability to Trypan Blue, led to a considerable decrease in the quantity of the glycoprotein associated with the cultured cells (Bloomfield *et al.*, 1977).

The precise localization of Tamm–Horsfall glycoprotein, at the subcellular level, in the tubular system of the foetal-, neonatal-(Sikri *et al.*, 1981a) and adult-(Sikri *et al.*, 1979) hamster kidney is

known: the glycoprotein is restricted to an association with the whole of the plasma membrane of cells of the thick ascending limb of the loops of Henle, and of those particular distal-tubular cells which are morphologically closely like those of the ascending limb. A similar distribution of the glycoprotein occurs also in the kidney of the rat (Hoyer *et al.*, 1979; Sikri *et al.*, 1982) and of man (Sikri *et al.*, 1981b). The probable implications of this distribution for the function of the kidney have been discussed (Sikri *et al.*, 1979; Foster *et al.*, 1979; Hoyer *et al.*, 1979).

The present work is concerned with identifying the subcellular localization of the related glycoprotein in three different substrains of cultured baby-hamster kidney cells, BHK-21/C13. Immunoelectron-microscopic procedures have been used for identifying the glycoprotein on these cells for the first time.

Experimental

Materials

Eagle's minimum essential growth medium, foetal-calf serum and tryptose/phosphate broth were purchased from Gibco-Biocult, Paisley,

§ To whom reprint requests should be addressed.

|| Present address: Department of Histochemistry, Royal Postgraduate Medical School, Du Cane Road, London W.12, U.K.

Renfrewshire, Scotland, U.K. DEAE-cellulose (DE-23) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K.; fluorescein-labelled sheep anti-(rabbit IgG) serum was from Wellcome Research Laboratories, Beckenham, Kent, U.K.; sheep anti-(rabbit IgG) serum and horseradish peroxidase-anti-peroxidase complex (PAP) were from Miles Laboratories, Stoke Poges, Slough, Berks., U.K.; and 3,3'-diaminobenzidine was from BDH Chemicals, Poole, Dorset, U.K. Samples of baby-hamster kidney cells, BHK-21/C13, substrain 2P, adapted to grow in suspension culture (Capstick *et al.*, 1966), were supplied by The Wellcome Foundation, Pirbright, Surrey, U.K., those of substrain 3P by The Wellcome Research Laboratory, Beckenham, Kent, U.K., and a third sample (BHK-21/ICRF) by the Imperial Cancer Research Fund Laboratory, Lincoln's Inn Fields, London W.C.2, U.K.

Methods

Hamster Tamm-Horsfall glycoprotein. The glycoprotein was isolated from hamster urine by the method of Dunstan *et al.* (1974), which includes gel filtration under dissociating conditions. Electro-

phoretic homogeneity on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was ascertained. Antiserum to Tamm-Horsfall glycoprotein was raised in rabbits (Sikri *et al.*, 1979), and IgG was isolated from the antiserum by a method (Bloomfield *et al.*, 1977) similar to that described by Sober & Peterson (1958). The freeze-dried product was dissolved in a volume of water equal to that of the serum from which it was derived, and the solution was stored in batches at -20°C . For use in the immunohistological experiments, the solution was diluted further (1:10, v/v) with phosphate-buffered saline, pH 7.3 (Dulbecco A buffer; Dulbecco & Vogt, 1954).

Growth and immunohistochemical staining of baby-hamster kidney cells. The cells were grown on glass surfaces, in the form of coverslips, for immunofluorescence microscopy, or in 500 ml medical 'flats' for immunoperoxidase experiments. In either case the cells were cultured in Eagle's minimum essential growth medium containing 10% (v/v) foetal-calf serum and 5% tryptose/phosphate broth at 37°C for 18–42 h.

Cells grown to confluence on coverslips were washed in 0.05 M-sodium phosphate buffer, pH 7.4, followed by 2–3 h fixation at 4°C in McLean &

Fig. 1. Indirect immunofluorescence method: strong fluorescence exhibited by a proportion of BHK-21/C13/2P cells

Fixation was done with periodate/lysine/paraformaldehyde. Magnification $\times 500$.

Fig. 2. BHK-21/C13/3P cells showing a positive reaction for Tamm-Horsfall-glycoprotein-like immunoreactivity in some of the cells

Immunoperoxidase method: $1\mu\text{m}$ section from resin-embedded cell pellet. Magnification $\times 440$.

Nakane's (1974) periodate/lysine/(2%, w/v) paraformaldehyde solution. The fixed cells were rinsed thoroughly with several changes of phosphate-buffered saline and processed for indirect fluorescence microscopy as described in detail elsewhere (Bloomfield *et al.*, 1977). The treated cells were examined with a Gillet and Siebert conference microscope fitted with a Zeiss FL epifluorescence attachment.

Cells grown in bottles were released from the glass by treatment with 0.02% neutralized EDTA in phosphate-buffered saline, pH 7.3. The suspension of cells was centrifuged (500g, 5min, 22°C). After the pelleted cells had been washed with the same buffer, they were fixed in periodate/lysine/paraformaldehyde solution (2-3h, 4°C) and processed for immunoperoxidase microscopy by using the peroxidase-anti-peroxi-

dase complex (Sternberger *et al.*, 1970; Sikri *et al.*, 1979).

After histochemical staining for peroxidase activity with 3,3-diaminobenzidine and H_2O_2 (10-15min; Graham & Karnovsky, 1966), the pelleted cells were post-fixed in 2% (w/v) OsO_4 for 1h and embedded in Taab's resin (Taab Laboratories, Emmey Green, Reading, Berks., U.K.). Sections (1 μm thick) were cut from the embedded material, mounted on glass slides and used for optical-microscopic observations. Ultrathin sections (0.05 μm) were cut on a Huxley ultramicrotome (Cambridge Instrument Co., Cambridge, U.K.), collected on uncoated 200-mesh copper grids and viewed, without further heavy-metal staining, with a Hitachi H300 electron microscope operating at 60kV.

In order to check for the specificity of the

Fig. 3. Low-power (magnification $\times 3500$) electron micrograph of pelleted BHK-21/C13/2P cells showing positively and negatively reacting cells

Note strong reactions in the numerous microvilli associated with the upper cell. Immunoperoxidase method.

reaction, control tests were done in which anti-(Tamm-Horsfall glycoprotein) antibody was omitted or was replaced by normal rabbit serum. Further control tests were done in which material was treated with anti-(Tamm-Horsfall glycoprotein) antibody which had been pre-absorbed with excess Tamm-Horsfall glycoprotein.

Results

Use of the immunofluorescence method indicated that, in two of the sublines of cells which were used (BHK-21/C13/3P and BHK-21/C13/2P), a proportion of the cells contained material cross-reactive with antibody raised against urinary Tamm-Horsfall glycoprotein (Fig. 1). The observations were similar to those reported previously (Bloomfield *et al.*, 1977).

Some of the cells, after use of the horseradish

peroxidase technique using the optical microscope (Fig. 2), showed a strong cytoplasmic reaction, but none in the nuclei. These findings by the two different methods are consistent in two respects, namely that only a relatively small proportion (5–10%) of the total cells was reactive, and the immunoreactive material was not associated with the nuclei.

The immunoelectron-microscopic method showed unequivocally that the protein was associated with the plasma membranes of the reactive cells, as demonstrated by the deposition of a dark reaction product (Figs. 3–5). Once again, only a small proportion of the total cells was reactive, with strongly stained plasma membranes clearly evident.

Fig. 4 shows that the reaction product was absent from part of the membranes where the two cells are in close apposition and, although

Fig. 4. Medium-power (magnification $\times 5500$) electron micrograph of one complete cell and part of another (BHK-21/C13/3P). Note the strong reaction associated with the plasma membranes, except at one place where the cells are in close apposition. Immunoperoxidase method.

Fig. 5. High-power (magnification $\times 12600$) electron micrograph of a part of a cell (BHK-21/C13/3P) showing Tamm-Horsfall-glycoprotein-like immunoreactivity associated with the plasma membrane and microvilli. The two elliptical profiles within the cytoplasm are either vesicles or invaginations of the plasma membrane. Immunoperoxidase method.

relatively uncommon, this was not an isolated example. In all instances it seems probable that the paired cells are the product of a recent cell division. Control preparations were in all cases negative, thus demonstrating the specificity of the immunofluorescence and immunoperoxidase reactions. The cell line BHK-21/ICRF yielded negative results by all the techniques used.

Discussion

The status of cells derived from a variety of organ explants, including kidney, and maintained by subculture over long periods has been a matter of considerable controversy. A decade or so ago it was widely held that in such cultures all the cells, or at least a very high proportion of them, were fibroblasts or endothelial cells or both (Franks & Wilson, 1970; Franks, 1972). However, subsequent evidence suggests that the dominant cell in the dog kidney cell line, MDCK, has epithelial-cell properties. This conclusion was reached from the

results of electrophysicochemical (Richardson *et al.*, 1981), biochemical, hormonal (Ishizuka *et al.*, 1978; Horster, 1980; Saier, 1981) and morphological (Rindler *et al.*, 1979) studies. McRoberts *et al.* (1981) concluded that 'MDCK cell monolayers are morphologically, functionally and biochemically similar to epithelia from the distal tubule of the kidney'. Although the morphological evidence for the last part of the statement does not seem totally compelling, there seems little doubt that MDCK cells are derived from some region, or regions, of the renal-tubular epithelium.

Although BHK-21/C13 cell lines have not been studied so intensively in this respect as have MDCK cells, there seems to be no reason *a priori* why they should not also contain cells of epithelial origin. This possibility is strengthened both by the earlier evidence from immunofluorescence studies (Bloomfield *et al.*, 1977) and by the present observations, which demonstrate a protein clearly related to, if not identical with, Tamm-Horsfall glycoprotein as being associated with a proportion

of BHK-21/3P and BHK-21/2P cells. But other explanations are possible.

The results obtained by immuno-electron-microscopy show that the protein is plasma-membrane-associated just as it is in the cells of the thick ascending limb of the loop of Henle and of the early distal convoluted tubule of the hamster (Sikri *et al.*, 1979), rat (Sikri *et al.*, 1982) and human (Sikri *et al.*, 1981b). The complete lack of reaction in BHK-21/ICRF cells remains unexplained.

We thank Professor Richard Creese for providing facilities for C. L. F. in the Department of Physiology, St. Mary's Hospital Medical School (SMHMC) during the latter stages of these investigations, Dr. John Williamson of the Department of Virology, SMHMC, for help with preparation of the cell cultures, The Wellcome Foundation, The Wellcome Research Laboratories and the Imperial Cancer Research Fund Laboratories, who kindly provided samples of the cultured cells.

References

- Bloomfield, F. J., Dunstan, D. R., Foster, C. L., Serafini-Cessi, F. & Marshall, R. D. (1977) *Biochem. J.* **164**, 41–51
- Capstick, P. B., Garland, A. J., Masters, R. C. & Chapman, W. C. (1966) *Exp. Cell Res.* **44**, 119–128
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182
- Dunstan, D. R., Grant, A. M. S., Marshall, R. D. & Neuberger, A. (1974) *Proc. R. Soc. London. Ser. B* **186**, 297–316
- Foster, C. L., Marshall, R. D., Sikri, K. L., Bloomfield, F. J. & Alexander, D. P. (1979) *J. Anat.* **128**, 417–418
- Franks, L. M. (1972) in *Ultrastructural Features of Cells and Tissues in Culture* (Töro, I. & Rappay, G., eds.), pp. 31–35, Akademiai Kiado, Budapest
- Franks, L. M. & Wilson, P. D. (1970) *Eur. J. Cancer* **6**, 517–523
- Graham, R. C., Jr. & Karnovsky, M. J. (1966) *J. Histochem. Cytochem.* **14**, 291–302
- Horster, M. (1980) *Klin. Wochenschr.* **58**, 965–973
- Hoyer, J. R., Sisson, S. P. & Vernier, R. L. (1979) *Lab. Invest.* **41**, 168–173
- Ishizuka, I., Tadano, K., Nagata, N., Niimura, Y. & Nagai, Y. (1978) *Biochim. Biophys. Acta* **541**, 467–482
- MacPherson, I. A. & Stoker, M. G. P. (1962) *Virology* **16**, 147–151
- McLean, I. W. & Nakane, P. K. (1974) *J. Histochem. Cytochem.* **22**, 1077–1083
- McRoberts, J. A., Taub, M. & Saier, M. H. (1981) in *Functionally Differentiated Cell Lines* (Sato, G., ed.), pp. 117–139, Alan R. Liss, New York
- Richardson, J. C. W., Scalera, V. & Simmons, N. L. (1981) *Biochim. Biophys. Acta* **673**, 26–36
- Rindler, M. J., Chuman, L. M., Schaffer, L. & Saier, M. H. (1979) *J. Cell Biol.* **81**, 635–648
- Saier, M. H. (1981) *Am. J. Physiol.* **240**, C106–C109
- Sikri, K. L., Foster, C. L., Bloomfield, F. J. & Marshall, R. D. (1979) *Biochem. J.* **181**, 525–532
- Sikri, K. L., Foster, C. L., Alexander, D. P. & Marshall, R. D. (1981a) *Biol. Neonate* **39**, 305–312
- Sikri, K. L., Foster, C. L., MacHugh, N. & Marshall, R. D. (1981b) *J. Anat.* **132**, 597–605
- Sikri, K. L., Alexander, D. P. & Foster, C. L. (1982) *J. Anat.* **135**, 29–47
- Sober, H. A. & Peterson, E. A. (1958) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **17**, 1116–1126
- Sternberger, L. A., Hardy, P. H., Cuculis, J. J. & Meyer, H. G. (1970) *J. Histochem. Cytochem.* **18**, 315–333
- Tamm, I. & Horsfall, F. L. (1950) *Proc. Soc. Exp. Biol. Med.* **74**, 108–114
- Tamm, I. & Horsfall, F. L. (1952) *J. Exp. Med.* **95**, 71–97