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Long-term Preservation of Renin-secreting Ability by Human Adult Juxtaglomerular Tumor Cells in Explant Culture

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Studies on cultured human renin(R)-producing tumors cells are few. In this work the R secretion by a human juxtaglomerular tumor (JGT) in various tissue culture models was evaluated by a new immunoradiometric assay. Freshly isolated JGT cells actively secreted total R (tR; about 70% of which is proR) into the perfusion media of very short-term cultures (tR concentration, 100–400 ng/ml/10^6 cells), independently of factors stimulating or inhibiting R output by normal JG cells. Primary monolayer cultures of the same JGT rapidly lost their tR-secreting capability and died by apoptosis within two months. Conversely, a JGT explant survived for up to 22 months *in vitro*. During the first year of culture, this explant increased in volume and generated, at 3- to 4-monthly intervals, several self-limited cellular outgrowths, from which it became detached. Meanwhile, tR secretion by the explant decreased very slowly, though its decline was transiently and partly reversed by various combinations of growth factors, hormones, a prostaglandin, and selenous acid added to either a serum-enriched or a synthetic medium. By the 12th month *in vitro*, tR secretion had faded away. Like the primary monolayers, the various explant outgrowths, once detached, stopped secreting tR and died in a few weeks. Hence, the preservation of a histiotypic relationship and the actions of several mitogenic and/or differentiating agents are essential for the long-term survival and the continuance of R secretion by human JGT cells *in vitro*.

Key words: Renin secretion — Hemangiopericytoma — Human — Kidney — Juxtaglomerular tumor

Although an infrequent cause of R[5]-dependent hypertension,[1,2] tumors of the JG apparatus, as well as other kidney or extra-renal R-secreting neoplasms, exhibit interesting features from the standpoint of human biology, and the availability of long-term *in vitro* cultures would be advantageous.[3–8] Although normal kidney R-secreting cells can be cultured,[9–11] their numbers are insufficient for most research purposes.[3] A few tissue cultures of JG neoplasms have been reported, but the isolated cells did not survive for longer than four weeks *in vitro*, where they swiftly lost their ability to process proR into R.[3–8,12,13] Endeavors to immortalize R-secreting cells by transfecting into them three mutants of the SV40 oncogenic virus led to the establishment of a human continuous JG cell line.[14,15] As to the non-JG tumors, neoplastic cells secreting proR could be cultured from nephroblastomas,[6,16] ovarian leiomyosarcomas[8] and virilizing tumors,[17] and the pulmonary metastases of an epithelioid sarcoma of vascular origin.[18] Renal (JG and non-JG) and extra-renal R-secreting tumor cells would be valuable for studies into: (a) the genetic mechanisms[19] and promoting agents eliciting these human neoplasms; (b) the factors regulating the expression of the R gene[9,20]; (c) the ordering of the synthesis, storage, and release of R[9,20]; and (d) the possibility of producing large amounts of R to be used, for example, to raise different monoclonal antibodies and thus to set up RIA and IRMA assays of R.[3,21–25]

The availability of an R-producing JGT surgically removed from a young hypertensive patient led us to investigate by means of short- and long-term tissue culture techniques the factors affecting the survival and the maintenance of tR secretion by this JGT. In order to evaluate the tR secreted *in vitro*, a new IRMA, herein described briefly, was set up and used. The results obtained show that the cultured JGT cells initially possessed an intense, autonomous tR-secreting activity that could be maintained for a long period by the preservation of an *in vivo*-like three-dimensional relationship amongst tumor cells coupled with the use of various mitogenic and differentiating agents.

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[5] Abbreviations: R, renin; AI, angiotensin I; ANP II, atrial natriuretic peptide II; BSA, bovine serum albumin; EBSS, Earle's balanced salt solution; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; IRMA, immunoradiometric assay; JG, juxtaglomerular; JGT, JG tumor; MEM, minimum essential medium; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$; PRA, plasma renin activity; proR, pro-renin; RIA, radioimmunoassay; T_3, triiodothyronine; tR, total renin (i.e., prorenin + renin); moAb, monoclonal antibodies.

MATERIALS AND METHODS

Patient A 23-year-old male was hypertensive, having a 160/120 mm Hg blood pressure. The analysis of his plasma electrolytes revealed a hypokalemia (2.5 mEq/liter). Also significantly increased were both his peripheral upright plasma R activity (PRA: 12 ng AI/ml/h; normal range, 1–6) and his urinary aldosterone (30 μg/24 h; normal values, 5–15). His PRA venous levels were as follows: right renal vein, 10; left renal vein, 6; and vena cava, 6 ng AI/ml/h. A parahilar mass within the right kidney was revealed by renal angiography. After the operation, both the clinical and the biochemical parameters of the patient reverted to the normal range. A detailed report of this case has been published elsewhere.[26]

Short-term perfusion culture A portion of the R-producing JGT was chopped into small pieces (2–3 mm^3) that were placed in 10 ml of EBSS containing collagenase type II from *Clostridium histolyticum* (2 mg/ml; activity, 1.5 U/mg; Sigma). The mechanical dispersion of the tumor cells was next carried out 4 times (20 min for each run) at 37°C in a Teflon dispersal apparatus. After the addition of 1000 KIU (kininogen-inhibitory units; Antagosan, Behring Ltd.), the cell suspension was spun down at 420*g* for 4 min. The isolated cells were resuspended in Biogel P2 (0.5 g; Bio-Rad). The cells-Biogel mixture was packed inside two plastic syringes (6.0×1.0 cm). Column perfusion was performed according to the method of Lowry and MacMartin[27] with minor modifications. Briefly, cells were perfused in a water bath at 37°C with 0.7 ml/min EBSS containing BSA (0.25% w/v) and gassed with a mixture of O_2 (95%) and CO_2 (5%). Four pulses of agents known either to stimulate or to inhibit R secretion were applied to both columns: they included norepinephrine (10^{-6} *M*), labetalol (10^{-6} *M*), rat ANP II (10^{-6} *M*), and dopamine (10^{-6} *M*).[28]

Long-term tissue cultures (A) Primary monolayers Part of the surgically removed JGT was decapsulated and cut into small pieces (1–3 mm^3). These tissue fragments underwent three runs of magnetic stirring (100 rpm), each lasting 20 min, in a tissue dissociating solution made up of 0.02% (w/v) trypsin (type II, crude, from porcine pancreas; activity, 1000 U/mg), collagenase type II, and hyaluronidase (type III, from sheep testis; activity, 375 U/mg) (all three enzymes from Sigma) at room temperature (18±2°C). The three supernatants thus collected were mixed with 20% (v/v) heat-inactivated (30 min at 56°C) adult bovine serum and next spun down at 4°C (50*g*×5 min). The packed cells were resuspended in Eagle's MEM (Flow Laboratories) fortified with 20% (v/v) heat-inactivated FBS (Flow), and seeded into three F75 (75 cm^2) flasks (Costar Ltd.), each containing 12 ml of growth medium, at a final density of about 10^6 cells/flask. The cultured cells were kept at 37°C in air containing 5% (v/v) CO_2. In some instances 30%FBS-MEM was used instead. As will be detailed in the "Results," no subculture of these cell monolayers could be performed.

(B) Explant culture One piece of the JGT tissue (2×2×3 mm) was not enzymatically dissociated, but directly seeded into an F25 (25 cm^2) flask (Costar) containing 4 ml of the 20%FBS-MEM, which was changed twice-weekly. This medium was used for the first months of culture. Thereafter, changes in the serum per cent fraction and additions of various agents to the medium were made both on an empirical basis and according to results gained with other types of cultured cells.[29–31] Thus, during the 5th and 6th months *in vitro*, an equimolar (10^{-10} *M*) mixture of EGF (Collaborative Research Inc.) with highly purified, crystalline glucagon and insulin (Eli Lilly Co.) was added to 30%FBS-MEM medium. During the 7th and 8th months, $PGF_{1\alpha}$ (1.0 ng/ml; Sigma) was instead added to 20%FBS-MEM. Thereafter, the synthetic $HiWo_5Ba_{2000}$ medium,[29,31] routinely fortified with EGF (10^{-9} *M*), FGF (10^{-9} *M*), insulin (10^{-10} *M*), hydrocortisone Na-succinate (10^{-7} *M*), T_3 (1.5 ng/ml), lysine-vasopressin (10^{-9} *M*), cholera toxin (choleragen; 10^{-8} *M*), and selenous acid (10^{-8} *M*) (all from Sigma, but insulin from Eli Lilly), was used instead, being also changed twice-weekly. All the JGT-conditioned media were collected and stored at −20°C to be subsequently assayed for their tR content.

Direct assay of tR by IRMA Two anti-R moAb, viz. R3-33-16 and R3-27-6, the attributes of which were detailed elsewhere,[32] were kindly provided by Dr. Ch. Heusser (Ciba-Geigy, Basel, Switzerland). Microtiter flexible PVC plates (Falcon 3911, Becton-Dickinson Ltd.) were coated with R3-27-6 moAb by adding to each well 150 μl of a 10 μg/ml dilution in coating buffer. After a 2-h incubation at 37°C in a moist chamber, the plates were washed 5 times with PBS, and the remaining protein-binding sites in the wells were blocked by the addition of 250 μl of PBS-BSA mixture. Such plates could be stored for several days at 4°C, provided that the wells contained enough (viz., 250 μl) PBS-BSA. The R3-36-16 moAb was iodinated according to the chloramine T method. For each iodination, 1.0 mCi of ^{125}I was allowed to react with 100 μg of this moAb. Free iodine was separated from the labeled moAb by means of an HPLC system. The mean specific activity of the iodinated moAb was 6.4 μCi/μg. The coated plates were washed thrice with PBS, and flicked over a sheet of paper towel, then PBS-BSA, 50 μl/well, was added. Next, 50 μl of sample or of standard R (range, 20–20000 pg/ml) was added. Each assay was performed in duplicate and a blank row of wells containing no R was always included. As a next step, 50 μl (about 10^5 cpm)/well of iodinated R3-36-16 moAb was added. The plates were gently shaken and

incubated first for 2 h at 37°C and subsequently for 30 min at 4°C in a moist chamber. After a thorough washing, the wells were cut out and counted in a gamma counter.

Enzymatic assay of R (PRA) The activity of R was expressed as the amount (ng/ml) of AI generated at pH 5.7 *in vitro* during a 3-h incubation in the presence of R- and of proR-"free" human plasma (acting as the source of R's substrate). In our system, the concentration of angiotensinogen was 21,000 ng/ml, thereby allowing the use of samples of a very tiny volume, as when the activity of R was evaluated with cell perfusion media at a substrate concentration equal to the K_m (viz., 1,400 ng/ml). The same procedure was used to measure tR after activation of proR with trypsin (40 μg/ml; Sigma) at 4°C for 45 min.

RESULTS

The granular JGT The surgically removed neoplasm was about 3.0 cm in diameter and well capsulated. Histopathologically, it resembled a benign renal hemangiopericytoma: however, immunocytochemically detectable R was present in the cytoplasm of a quite remarkable proportion of its cells (Fig. 1).[26]

The assay of tR by IRMA The sensitivity of the IRMA presently used to evaluate the concentration of tR in both the short- and the long-term tissue culture media was 18 ± 7 pg/ml (mean $\pm$ 1 SD). It should be stressed that by this method tR was found to be virtually absent (i.e., values close to 0) in control media of either kind. The reproducibility of this IRMA was sufficiently accurate for most comparative purposes, as its coefficients of variability were: (i) intra-assay: 12.1 (perfusion media) and 18.5% (tissue culture media); and (ii) inter-assay: 15.9 (perfusion media) and 21.3% (tissue culture media). The IRMA's accuracy was confirmed by the finding that the dilution curve of a standard sample of human R paralleled the curve obtained by serially diluting tumor-conditioned tissue culture media (data not plotted).

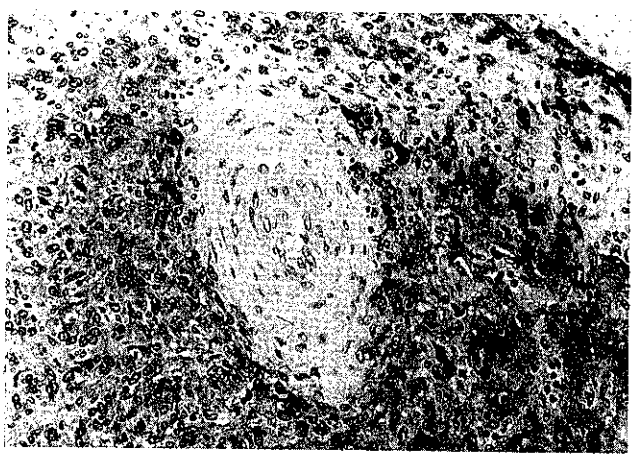

Fig. 1. Histopathology of the human JGT studied. Although the tumor resembled a renal hemangiopericytoma, a conspicuous fraction of its cells contained R in their cytoplasm, as shown by the use of anti-human-R monoclonal antibodies (PAP-diaminobenzidine technique; counterstained with hematoxylin). The R-negative perivascular area in the center of the field is clearly distinguishable. ×315.

The secretion of tR by freshly isolated JGT cells in short-term perfusion culture The above-mentioned IRMA was used, in conjunction with the classic enzymatic assessments of PRA before and after trypsin activation, to evaluate the secretion of tR by JGT cells enzymatically isolated, cultured and perfused within 5 h after the operation. Under these conditions, the JGT cells were found to be capable of a large and sustained secretion of tR, about 70% of which was in the form of its precursor proR (Fig. 2). The steady-state level of this activity was not changed by the sequential addition to the perfusing media of several agents that stimulate or inhibit R secretion by normal JG cells, namely norepinephrine, dopamine, ANP II, and the β- (and α-) adrenergic blocker labetalol[28] (Fig. 2). The tR concentration in the perfusion media assayed by IRMA ranged between 200 and 400 ng/ml/10^6 cells, of which the active R and the inactive proR, measured by PRA, amounted to 47–95 ng/ml/3 h and 150-275 ng/ml/3 h, respectively.

The JGT cells in long-term tissue cultures (i) Cell survival, growth and apoptosis Although the culture of primary nonconfluent monolayers of isolated JGT cells was initially successful, such cells neither displayed any mitotic activity nor could be induced to multiply by increasing up to 30% (v/v) the FBS fraction in the MEM. By the 5th week *in vitro*, typical apoptotic figures became detectable and cell numbers declined steadily in the monolayers, with total extinction at the 8th week. This apoptotic process was not due to any toxic effect exerted by the FBS or the MEM, as the same batches of MEM and serum fully supported the concurrent growth both of a JGT tissue explant (see below) and of other cell types (e.g., hepatocytes) *in vitro*.[29]

Conversely, a JGT tissue explant promptly attached to the surface of a T25 flask and thrived. By the second week, a slow outgrowth erupted from its edges, made up of three distinct cell types, i.e. (i) thin, agranular spindle-shaped cells; (ii) cells endowed with numerous, dark cytoplasmic granules and tiny vacuoles; and (iii) cells having an eccentric nucleus and a complex channel-like cytoplasmic structure (Fig. 3). Type (ii) cells prevailed in the outgrowth's innermost region, while the three cell types were equally represented at its periphery. These

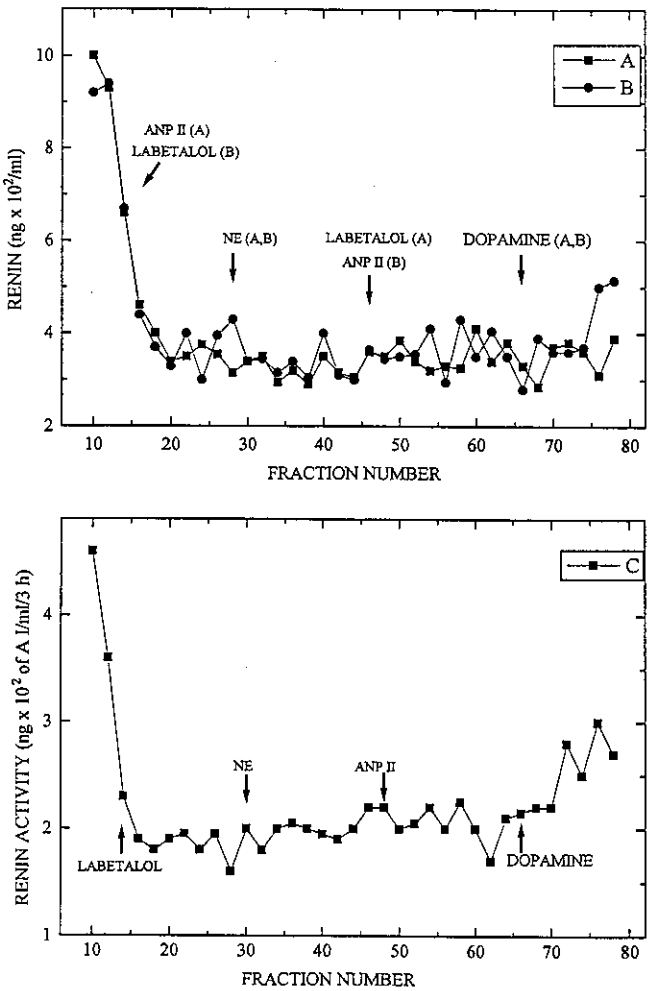

Fig. 2. The renin secretion into the perfusion effluent by short-term cultures of JGT cells. Upper panel. Estimates of R by IRMA in the effluents from two distinct columns, namely 1 (A) and 2 (B). Lower Panel. (C) The R activity assessed after the addition of human angiotensinogen to the same (B) samples shown in the upper panel. The arrows indicate the times at which the various pharmacological stimuli were applied with a different sequence for each column (as indicated within brackets in the upper panel). ANP II, atrial natriuretic peptide II; NE, norepinephrine; AI, angiotensin I.

cells showed no proliferative activity. The outgrowth ceased enlarging by the 10th week *in vitro*, having a mean diameter of about 30 mm. Concurrently, the JGT explant had been appreciably increasing in size, assuming an oval shape (radii, 3.0×4.5 mm) by the end of the 4th month. Then, this enlargement caused the detachment of the explant from both the flask's surface and the cellular outgrowth (which itself stayed in place). Once freed, the explant was transferred into a second T25 flask containing MEM plus an equimolar mixture of EGF, glucagon, and insulin, and FBS (i.e., 30% v/v). There, after about a week, it became attached to the flask's surface and slowly generated a second outgrowth made up of type (i) and type (ii) cells. By the end of the 23rd week *in vitro*, having grown even rounder, the tumor explant again freed itself completely, and was then moved to a third flask. There, under the stimulus of the powerful mitogen $PGF_{1\alpha}$[30] in 20%FBS-MEM medium, it elicited a third outgrowth of type (i) cells. At the end of the 33rd week, the JGT explant once more loosened itself and was transferred into a fourth flask, where it was kept in the synthetic $HiWo_5Ba_{2000}$ medium[29,31] fortified with hormones (T_3, insulin, hydrocortisone, vasopressin), growth factors (FGF, EGF, choleragen), and the anti-oxidant selenous acid. A fourth outgrowth of cells, equally distributed among the above-mentioned three types, formed and reached a maximum diameter of about 12 mm. Eventually, by the start of the 11th month in culture, the explant released itself from this last outgrowth. Although moved into another flask, it produced no further cellular sheet, not even when, after 56 weeks *in vitro*, it was cautiously cut into four pieces by means of ophthalmic scalpels. These tissue pieces lingered on for a further 12 months *in vitro* metabolizing the fortified $HiWo_5Ba_{2000}$ medium at a rate that declined very slowly. At termination, an immunohistochemical survey revealed that the pieces were composed of intertwined whorls of collagen fibers and poorly differentiated stromal cells that contained no detectable R (not shown).

The four cellular outgrowths left behind by the JGT explant behaved just as the primary monolayers of isolated JGT cells: they underwent extinction by progressive apoptosis within 8 weeks.

(ii) The secretion of tR into the media of long-term tissue cultures The assay of the tR content of the culture media by IRMA revealed that after 28 days *in vitro* the primary monolayers of isolated JGT cells no longer secreted any measurable amount of this peptide (data not plotted).

In contrast, the JGT explant kept secreting R for 11 consecutive months *in vitro*, during the first 4 of which this activity remained remarkably high and steady (Fig. 4). After a transient drop, coincident with the first shift to a new flask, the explant's tR secretion surged again between the 18th and 22nd week in a 30%FBS-MEM containing EGF, glucagon, and insulin (Fig. 4). Thereafter, between the 23rd week and the 30th week *in vitro*, the tR concentration of the media started dwindling slowly. Yet, after 6 weeks of exposure to a 20%FBS-MEM medium containing $PGF_{1\alpha}$, the tR secretion stopped falling and steadied at about 1/10th of its initial level (Fig. 4). The shift to the synthetic $HiWo_5Ba_{2000}$ medium fortified with several hormones, growth factors and selenous acid revived, though transiently, the tR secretion between the 35th and 38th weeks (Fig. 4).

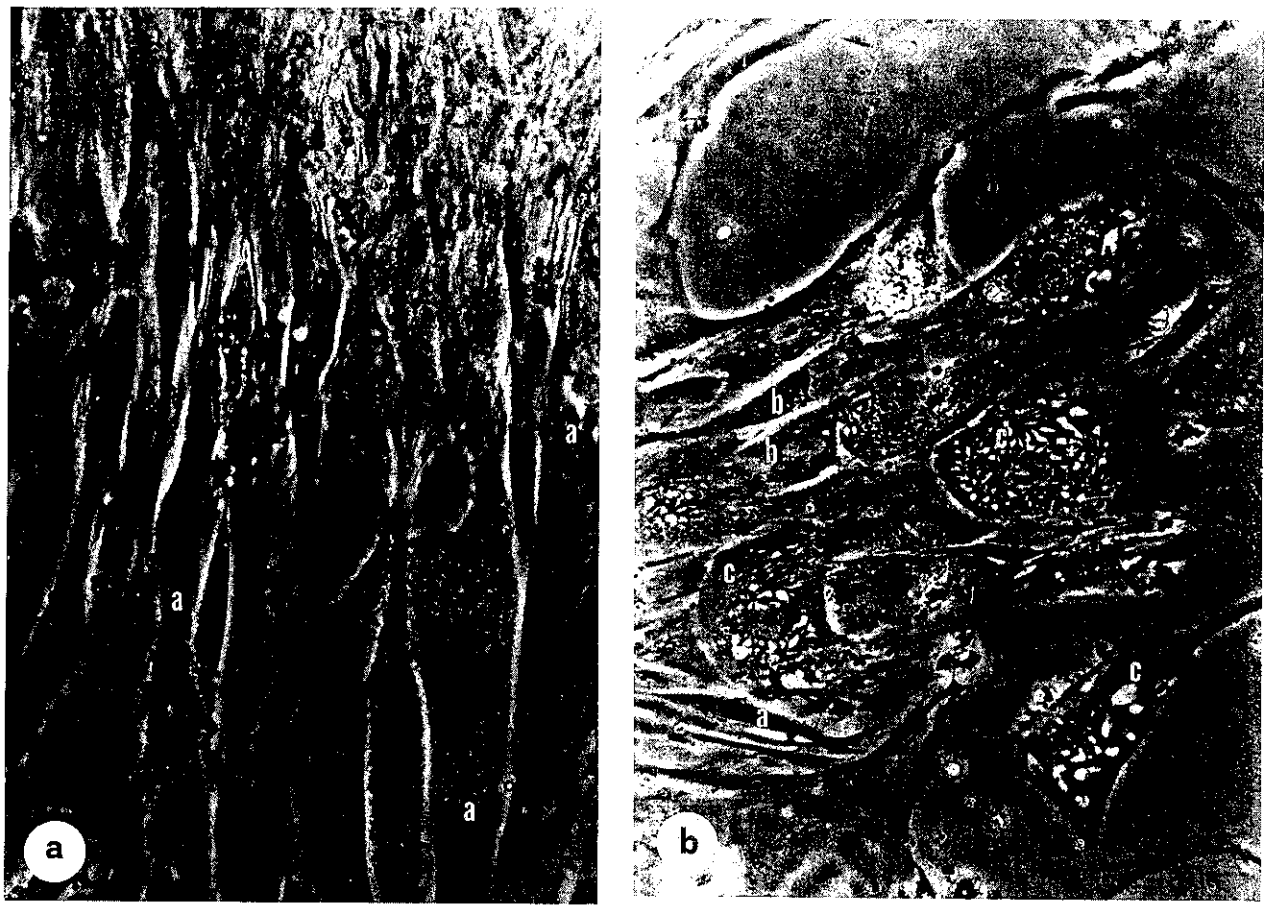

Fig. 3. The morphology of cells grown out of the JGT explant. (a) An intermediate area of the first cell outgrowth after 2 months in culture. From top to bottom the numbers of overlapping cell layers decrease as the distance from the explant's body increases. a, Type (ii) cells, endowed with cytoplasmic dark granules and tiny vacuoles, predominate in this region. Phase contrast microscopy. ×1000. (b) Part of the periphery of the same outgrowth as in (a). Three kinds of cells are recognizable viz., a, type (i) thin, agranular, spindle-shaped cells; b, type (ii) polygonal cells, endowed with numerous cytoplasmic dark granules and tiny vacuoles; and c, type (iii) cells, having an eccentric, oval nucleus and a wide polygonal cytoplasm, within which several channel-like anastomosing structures are visible. Phase contrast microscopy. ×525.

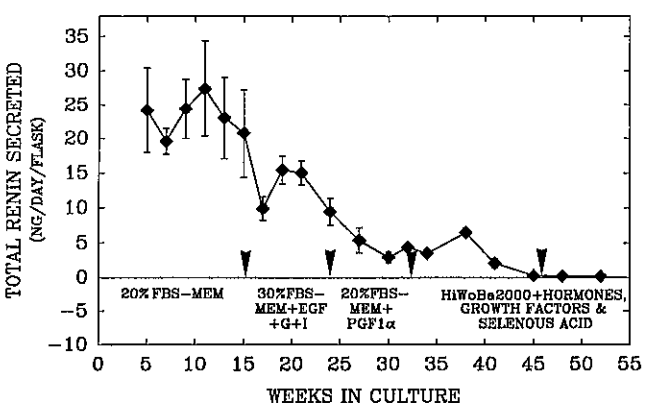

Fig. 4. The time-related decline of the R-secreting ability by the JGT explant kept in various tissue culture media (see "Materials and Methods" for details). The growth media were changed twice-weekly. The points are means±SD of the daily rate of tR secretion at 2-weekly intervals. If no SD is shown, it was smaller than the symbol used to represent the point. The arrowheads indicate the time points at which the enlarging tumor explant detached itself from the flask's surface and the surrounding cellular ontgrowth and was moved to another flask. G+I, equimolar (10^{-10} *M*) mixture of glucagon and insulin.

Eventually, by the 11th month *in vitro*, the secretion of tR by the JGT explant dropped to insignificant values (Fig. 4), never to rise again during the subsequent 12 months in culture (data not shown).

As with the primary monolayers, the cellular outgrowths left behind by the self-disjoining parent JGT explant exhibited a very short-lived, nearly insignificant ability to release tR into the medium (data not plotted).

DISCUSSION

The use of a direct IRMA enabled us to monitor the tR in short- and long-term tissue culture media, even when the concentration dropped to levels undetectable by the more conventional PRA. Moreover, the observed parallelism between IRMA's standard curve and the curve plotted by serially diluting the tissue culture media suggests that the JGT cells we examined were secreting a form of R immunologically identical with pure human R.

The short-term perfusion culture of isolated JGT cells was aimed at revealing the basal, *in vivo*-like features of their secretory activity. On the other hand, this is the first report, to our knowledge, of such a long-term maintenance of tR scretion by human JGT cells cultured without deliberately modifying their genomic complement. Previously, R-secreting cell lines were established either by directly transfecting mutants of the SV40 virus into JGT cells[14, 15] or by isolating them from kidney tumors of transgenic mice containing an R-promoter-SV40-T-antigen fusion transgene.[33]

The present findings demonstrate that the long-term explant culture of R-secreting JGT cells offered a distinct advantage over the monolayer culture model, providing a far superior survival and functioning of JGT cells. Although our observations concern a single JGT, they should be generally applicable to R-producing kidney tumors. In fact, our results on the monolayer cultures of JGT cells are in keeping with previous reports on the behavior of both normal and tumor JG cells in such settings.[3–8, 13, 34–37] Hence, they support the validity even for human JGT cells of Minuth's contention[37] that the upkeep of the synthesis and secretion of R by JG cells *in vitro* depends upon their protracted contact with specific, albeit as yet unidentified protein(s) of the renal extracellular matrix. However, our results further indicate that the survival and growth of JGT cells *in vitro* are also strongly conditioned by the maintenance of histiotypic interrelationships that closely resemble those *in vivo* and that may favor the occurrence of critical interactions between different cell types within the tumor. The total subversion of such interactions in the primary monolayer cultures and in the several explant-"orphaned" outgrowths of JGT cells would account for their rapid loss of the R-secreting ability and their ensuing apoptosis.

Just as in previously reported instances[2–8, 33] a prevailing proR secretion was characteristically effected by the JGT presently investigated. This incomplete processing of proR into active R (the so-called "constitutive pathway"[8]) has been ascribed to the anomalous lack of specific maturation enzymes[3] and/or to an abnormal degree of glycosylation of proR[38] in the neoplastic JG cells. Further, it is noteworthy that the isolated R-secreting JGT cells in short-term cultures behaved just as autonomously as they would have *in vivo*, where no response by JGT's to physiological or pharmacological stimuli is usually observed.[2–8, 26, 39]

In the past, the best long-term cultures of normal kidney R-containing cells have been obtained when 10% FBS, platelet-derived growth factor and glycyl-histidyl-lysine were added to Dulbecco's MEM.[37] The present observations show that, besides fetal serum factors, various agents positively affected the survival, growth and R-secreting activity of the JGT cells, possibly in cooperation with some necessary, though as-yet-unidentified explant-produced autocrine agent(s). Although such factors both maintained the differentiation and stimulated the proliferation of R-producing cells inside the JGT explant, it is difficult to make clear-cut distinctions in their mechanisms of action. Conceivably fetal serum factors, EGF, glucagon, vasopressin, and choleragenfavored cell differentiation by enhancing the *de novo* synthesis of intracellular cyclic AMP, which decreases cytosolic free Ca^{2+} and thereby induces the synthesis and secretion of R by normal and neoplastic JG cells.[11, 15, 16, 20, 28, 33, 40–44] On the other hand, various lines of evidence indicate that prostaglandins are involved in the control of R secretion.[45] However, since it took 6 weeks of $PGF_{1\alpha}$ exposure to stop the decline of this activity in the explant culture, we surmise that the predominant effect of this agent was to stimulate the proliferation of R-producing cells,[32] thereby laying the foundation for the last, transient increase in tR-secretion subsequently brought about by a complex panel of agents added to a synthetic medium (cf. Fig. 4).

In conclusion, the present data show for the first time that it is possible to maintain human functioning JGT tissue in long-term explant cultures in a medium as simple as FBS-MEM. Moreover, they also suggest that a finely tuned combination of factors stimulating the production of cyclic AMP and/or the proliferation of JGT cells may bring about even more rewarding results.

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