In vitro culture on Matrigel favors the long-term maintenance of rat zona glomerulosa-cell differentiated phenotype

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Abstract. Zona glomerulosa (ZG) cells cultured on plastic within few days dedifferentiate losing their capacity to secrete aldosterone (ALDO) in appreciable amounts. Evidence indicates that extracellular matrix modulates the secretory behavior of adrenocortical cells cultured *in vitro*. Hence, we compared the morphology and function of rat ZG cells grown on plastic and Matrigel basement membrane matrix (hereinafter Matrigel) for up to 12 days. At day 3, no significant differences were observed between cells cultured on plastic and Matrigel. Starting from day 6, ZG cells cultured on plastic lost their ultrastructural differentiated features (mitochondria with tubular cristae, smooth endoplasmic reticulum cisternae and lipid droplets), exhibiting a fibroblast-like appearance. The mRNA expression of the main steroidogenic enzymes, as evaluated by real-time polymerase chain reaction, the baseline secretion of ALDO and other post-pregnenolone hormones, as evaluated by high pressure liquid chromatography, and the secretory response to ACTH, angiotensin-II and K^+, as evaluated by radioimmunoassay, displayed a time-dependent decrease. Matrigel was found to maintain unchanged both the ultrastructure and the expresion of steroidogenic enzymes of ZG cells until day 12 of culture. Baseline and agonist-stimulated steroid-hormone secretion decreased with the duration of culture on Matrigel, but was always higher than that of ZG cells grown on plastic. Hence, our study clearly indicates that the culture on Matrigel favors the maintenance of rat ZG-cell differentiated phenotype, allowing the conclusion that this technique is suitable for long-term *in vitro* investigations.

Introduction

Primary culture of adrenocortical cells is a widely used experimental model for the study of steroid-hormone secretion and its regulation. However, the limited life-span of adrenocortical cells in culture, due to their rapid dedifferentiation into fibroblast-like cells (reviewed in ref. 1), prevents the use of primary cultures in long-term studies. This is particularly true in the case of zona glomerulosa (ZG) cells, which within 48-72 h of *in vitro* growth lose their capability to secrete aldosterone (ALDO).

The use of stabilized and immortalized adrenocortical cell lines could overcome these difficulties (reviewed in ref. 2). Unfortunately, the presently available cell lines, that are derived from adrenocortical tumors, do not appear to be a very suitable model for investigating adrenal gland physiology, inasmuch as their ultrastructural features do not reflect those of normal adrenocortical cells (1,3,4) and their steroidogenic capacity is abnormal (murine Y1 cells do not secrete corticosterone) (2), almost completely absent (human SW-13 cells) (3) or very low (human H295 cells produce only small amounts of ALDO) (2,4). A recently described cell line obtained from mice harboring a temperature sensitive large T-antigen gene of simian virus 40 does not express ALDO synthase (5). Moreover, despite the fact that the rat is the most common species used in adrenocortical studies, rat adrenocortical cell lines are not yet available. Hence, the need of a primary culture technique allowing the long-term preservation of the differentiated phenotype of rat adrenocortical, and especially ZG, cells is compelling.

Numerous lines of evidence indicate that extracellular matrix plays an important role in modulating specific cell functions (reviewed in ref. 6), including the secretory behavior of human adrenocortical cells cultured *in vitro* (7). Matrigel basement membrane matrix (hereinafter Matrigel) is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma (8), which was found to be effective for the attachment and differentiation of several types of cells, including hepatocytes, and Sertoli, thyroid and endothelial cells (9-12). Based on these findings, we thought worthwhile to investigate the effects of the culture on Matrigel, as compared to the conventional growth on plastic, on the morphology and function of rat ZG cells.

Materials and methods

Animals and reagents. Adult male Sprague-Dawley rats were purchased from Charles-River (Como, Italy), and the protocol of the experiments was approved by the local Ethics Committee

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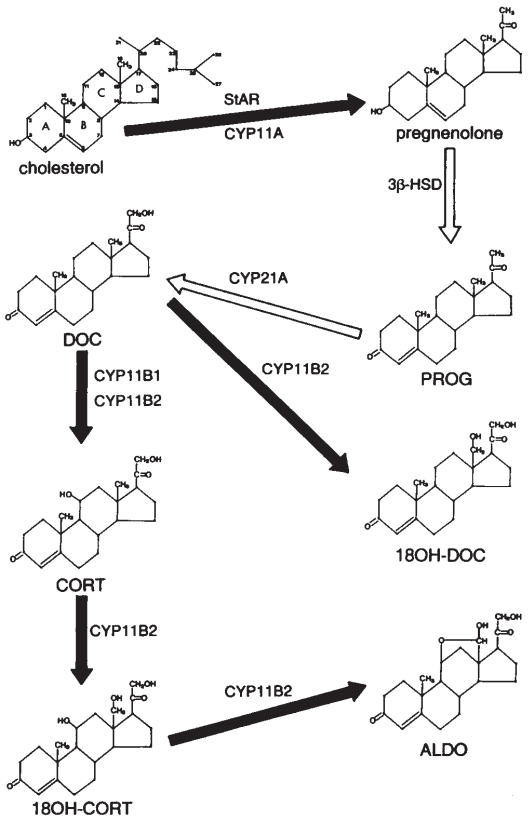

Figure 1. Pathway of ALDO synthesis in rat ZG cells. Steps occurring in mitochondria and SER are indicated by solid and void arrows, respectively.

for Animal Studies. Growth factor reduced Matrigel was obtained from BD Biosciences (Bedford, MA), and iTaq DNA polymerase from Bio-Rad Laboratories (Milan, Italy). Collagenase and deoxyribonuclease were provided by Worthington Biochemical Corp. (Lake Wood, NJ), tritiated aldosterone and corticosterone by Amersham (Aylesbury, UK), and ACTH and angiotensin-II (Ang-II) by Phoenix Pharmaceuticals (Belmont, CA). Glutaraldehyde and osmium tetroxide were purchased from Serva (Heidelberg, Germany), and Epon-812 resin from Electron Microscopy Sciences (Fort Washington, PA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), anti-aldosterone and anti-corticosterone antisera, antibiotics, and all other chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO).

Tissue culture. Rats were sacrificed by cervical dislocation, and adrenal glands were promptly removed and decapsulated to separate ZG. Dispersed ZG cells were obtained by sequential enzymatic digestion (collagenase-I, 2 mg/ml and deoxyribonuclease-I, 0.1 mg/ml in DMEM) and mechanical disaggregation. The purity of ZG-cell preparations, as checked by phase microscopy, was higher than 92%. Dispersed cells were seeded at a density of $2x10^4$ cells/cm^2 into 24-well plates, polystyrene dishes either uncoated or precoated with Matrigel (1 mg/ml). Cells were cultured at 37˚C in DMEM (supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 U/ml amphotericin) in an atmosphere of 95% air-5% CO_2 (13). Medium

Table I. Real-time PCR primers and PCR products.

Primer	Sequence	Annealing temp. (˚C)	Product size (bp)	Accession number
StAR				
Sense	5'-GCATACTCAACAACCAGGAAGGCT-3'			
Antisense	5'-TGCGGTCCACCAGTTCTTCATAGA-3'	60	159	NM_031558
CYP11A				
Sense	5'-AACTAAGACCTGGAAGGACCATGC-3'			
Antisense	5'-GGTACTTGCTGAAGTCTCGCTTCT-3'	60	116	NM_017286
3ß-HSD				
Sense	5'-CCACACGGCTTCTGTCATGGAT-3'			
Antisense	5'-GCAGTAGATGAAGGCTGGCACA-3'	60	130	NM_017265
CYP21A				
Sense	5'-CAAGCAGGACAGCACTTTGC-3'			
Antisense	5'-GGGGAAGAACCTGAGAAAGG-3'	60	118	NM_057101
CYP11B1				
Sense	5'-TGCTGGAGAATGTTCATGGAAG-3'			
Antisense	5'-ACTCTGTGCTACCATCTCGG-3'	56	168	RNCP450B
CYP11B2				
Sense	5'-AGGTGCGTCAGAATGCTCG-3'			
Antisense	5'-TAGTGCTGCCACAATGCCAC-3'	66	356	NM_012538
GAPDH				
Sense	5'-GGGCTGCCTTCTCTTGTGAC-3'			
Antisense	5'-CGCCAGTAGACTCCACGACA-3'	60	243	NM_017008

was collected at the 3rd, 6th, 10th and 12th day of culture (time 0), as well as 180 min before and 180 min after time 0, and new fresh medium was added. During the second 180-min incubation, the medium of some cultures contained 10^{-8} M ACTH, 10^{-8} M Ang-II or 10^{-2} M K^+. Collected media were stored at -80˚C until hormone assay.

Electron microscopy. Freshly dispersed ZG cells and control cultures were fixed in 3% phosphate-buffered glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Epon-812 (3). Thin (60-80 nm) sections were cut with an LKB SuperNova ultramicrotome (Reichert-Jung, Vienna, Austria), counterstained with lead-hydroxide, and observed in a Hitachi H-300 transmission electron microscope.

Reverse transcription (RT) real-time polymerase chain reaction (PCR). Freshly dispersed and control ZG cells cultured for 3, 6 and 10 days were harvested and frozen. Total RNA was extracted, and reverse transcribed to cDNA (14-16). The RT-reaction solution (1 μl diluted to 12 ng/ml) was added to a mixture (final volume, 25 μl), containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 3 mM $MgCl_2$, 0.8 mM dNTPs, and 25 U/μl iTaq DNA polymerase (17,18). PCR was performed in a Bio-Rad I-Cycler iQ Detection System, using the following protocol: denaturation program (95˚C for 3 min), 35 cycles of two steps of amplification (95˚C for 15 sec and annealing for 30 sec), and melting curve (60-90˚C with a heating rate of 0.5˚C/10 sec). The mRNA expression of the following steroidogenic enzymes was assayed: steroidogenic acute regulatory protein (StAR), cytochrome P_{450} (CYP) side-chain cleavage enzyme (CYP11A), 3ß-hydroxysteroid dehydrogenase (3ß-HSD), c21-hydroxylase (CYP21A), c11-hydroxylase (CYP11B1) and ALDO synthase (CYP11B2) (Fig. 1). Primer sequences, annealing temperature and accession numbers are shown in Table I. During the exponential phase, the fluorescence signal threshold was calculated, and the fraction number of PCR cycles required to reach the threshold (cycle threshold, Ct) was determined. Ct value decreased linearly with increasing input target quantity, and were used to calculate the relative mRNA expression, according to a mathematical quantification model (19). The specificity of amplification was tested at the end of each run by real-time PCR melting analysis, using the I-Cycler iQ software 3.0. All samples were amplified in duplicate and compared with the respective control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference to normalize data.

Steroid-hormone assay. The concentration of the following post-pregnenolone steroid hormones in the incubation media was assayed by quantitative high pressure liquid chromatography (HPLC) (20): progesterone (PROG), 11-deoxycorticosterone (DOC), 18-hydroxy-11-deoxycorticosterone (18OH-DOC), corticosterone (CORT), 18-hydroxy-corticosterone (18OH-CORT) and ALDO (Fig. 1). Steroids were detected by UV absorbance at 240 nm wavelength and identified by comparison of their retention times with those of the standards. A good separation between the steroid hormones assayed was obtained (Fig. 2), and the final recovery of steroids was 80-85%. Quantification of steroid hormones was based

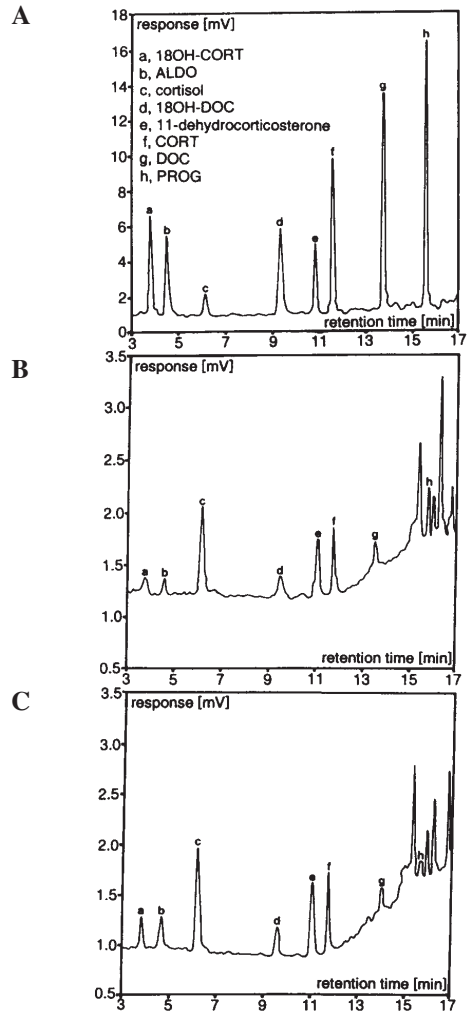

Figure 2. HPLC separation of steroid-hormone standards (A), and exemplary chromatograms of steroid hormones released by ZG cells cultured for 3 days on plastic (B) and Matrigel (C). Cortisol is the internal standard.

on peak area measurement; the sensitivity of our assay system was 1 pmol/ml, and the response of the detector was satisfactorily linear over the range of 1-1000 pmoles and directly proportional to the mass of steroid hormone injected. Intra- and interassay CVs were 5.6 and 7.7%, respectively. CORT and ALDO concentrations were also measured by radio-immuno assay (RIA), as previously detailed (21), using [1,2,6,7-^{3}H]-ALDO and [1α,2α(n)-^{3}H]-CORT (SA, 1.96 TBq/nmol) and antisera developed in rabbit. ALDO RIA: sensitivity, 5 pg/ml. Cross-reactivity: ALDO, 100%; and 17-iso-ALDO and other steroids, <0.1%. Intra- and interassay CVs, 5.3 and 7.1%, respectively. CORT RIA: sensitivity, 50 pg/ml. Crossreactivity: CORT and cortisol, 100%; DOC and PROG, 2%; and other steroids, <0.001%. Intra- and inter-assay CVs, 7.5 and 8.8%, respectively.

Statistics. Data were expressed as means ± SEM of four separate experiments. The statistical comparison was done by ANOVA, followed by Duncan's multiple range test.

Results

Morphology. Phase microscopy showed that ZG cells grown for 3 days on either plastic or Matrigel displayed a polygonal

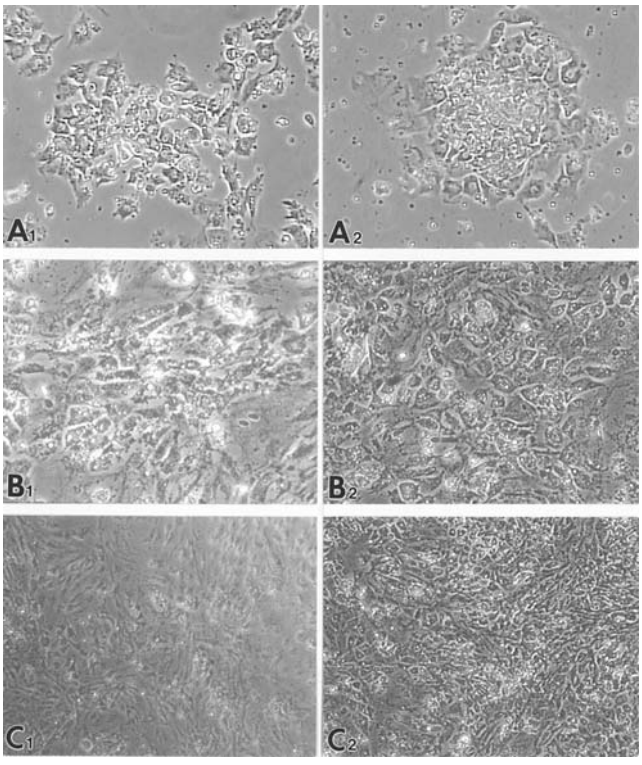

Figure 3. Phase-contrast micrographs of rat ZG cells cultured for 3 (A), 6 (B) and 12 days (C) on plastic (A1, B1 and C1) or Matrigel (A2, B2 and C2). At day 3 of culture on both plastic and Matrigel (A1 and A2), ZG cells display a polygonal shape and contain abundant clear vacuoles (lipid droplets). By prolonging the culture on plastic, ZG cells progressively assume an elongated fibroblast-like appearance (B1 and C1), while when grown on Matrigel ZG cells keep the epithelial-like morphology (B2 and C2). Magnification x55.

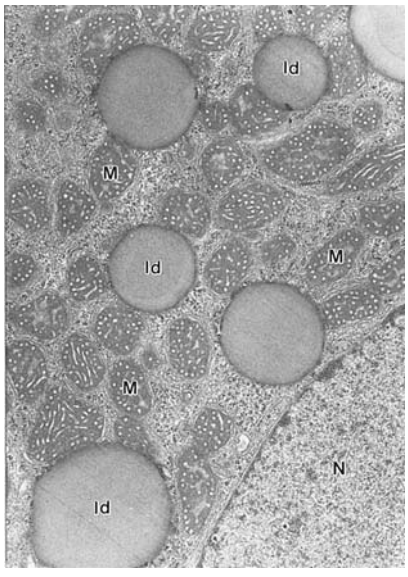

Figure 4. Electron micrograph of a freshly dispersed rat ZG cell. Mitocondria (M) contain tubular cristae, lipid droplets (ld) are numerous, and SER and RER cisternae, as well as free ribosomes, are present in the cytoplasm. N, nucleus. Magnification x9600.

shape and contained several lipid droplets (Fig. 3A). After 6, 10 or 12 days of culture on plastic, ZG cells exhibited a fibroblast-like elongated shape and were deprived of lipid

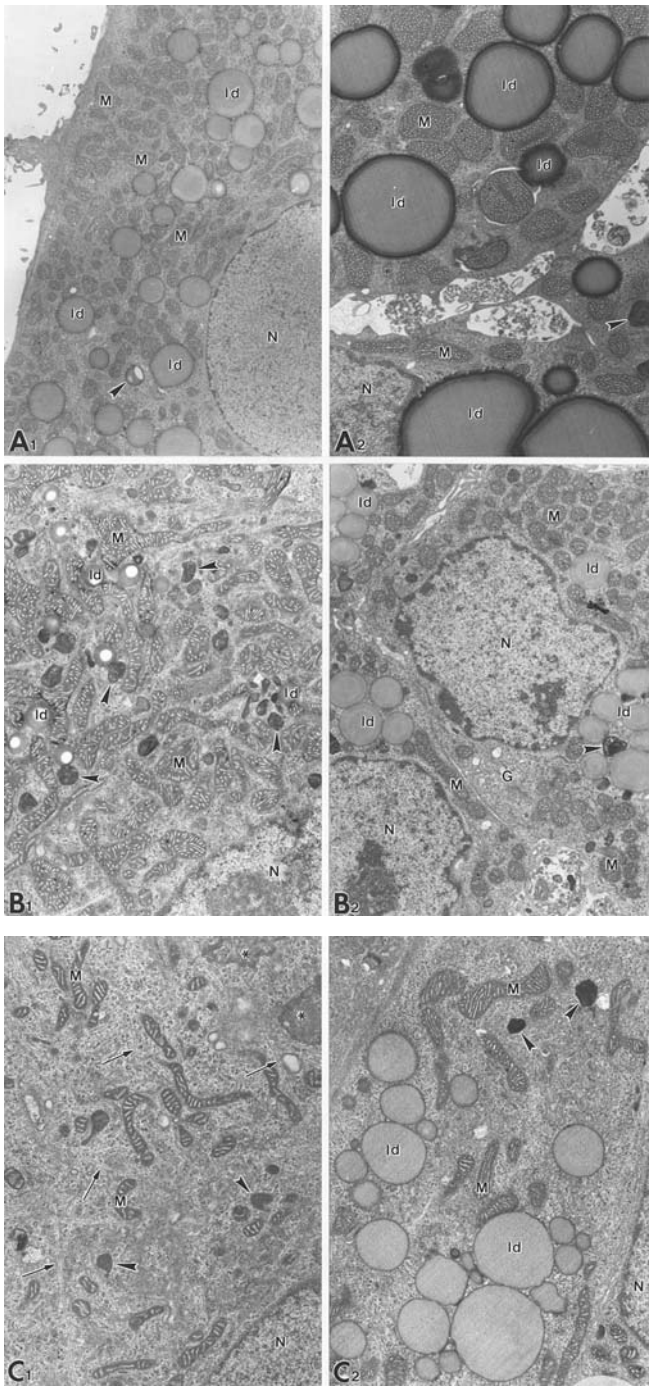

Figure 5. Low power electron micrographs illustrating the ultrastructural features of rat ZG cells cultured for 3 (A), 6 (B) and 12 days (C) on plastic (A1, B1 and C1) or Matrigel (A2, B2 and C2). After a 3 day culture on both plastic and Matrigel (A1 and A2), ZG cells retain the morphology of freshly dispersed cells: mitochondria (M) display tubular cristae and lipid droplets are abundant. After a 6 day culture on plastic (B1), ZG cells show irregularly-shaped elongated mitochondria (M) endowed with laminar cristae, few small lipid droplets (ld) and numerous dense bodies (arrow heads) of probable lysosomal nature. Conversely, ZG cells grown on Matrigel (B2) are still provided with mitochondria (M) with tubular cristae, several lipid droplets (ld) and a well-developed juxta-nuclear Golgi apparatus (G). After a 12 day culture on plastic (C1), ZG cells lose their differentiated morphological phenotype: mitochondria (M) are slender with few lamellar cristae, lipid droplets are absent, and dense bodies (arrowheads) are abundant; secondary lysosomes (asterisks) and several microtubules (arrows) can be observed. In contrast, when grown on Matrigel for 12 days (C2), ZG cells, although displaying poorly differentiated mitochondria (M), still possess abundant lipid droplets (ld) and SER and RER profiles. N, nucleus. Magnification: (A1), x3350; (A2), x5900; (B1), x5900; (B2), x4350; (C1), x5900; (C2), x5600.

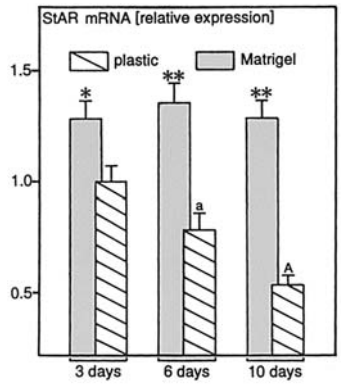

Figure 6. StAR mRNA expression in rat ZG cells cultured on plastic or Matrigel. Data are expressed as percent change from StAR expression in cells grown for 3 days on plastic (taken equal to 1). Bars are means ± SEM (n=4). *P<0.05 and **P<0.01 from the respective plastic value; aP<0.05 and AP<0.01 from the respective 3-day value.

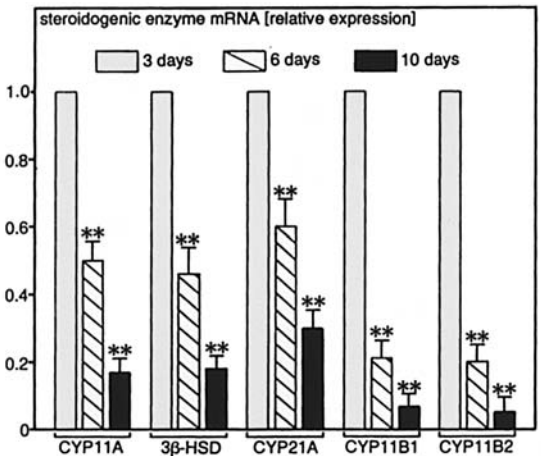

Figure 7. Steroidogenic-enzyme mRNA expression in rat ZG cells cultured on plastic. Data are expressed as percent change from the expression in cells grown for 3 days (taken equal to 1). Bars are means ± SEM (n=4). **P<0.01 from the respective 3-day value.

droplets (Fig. 3B1 and C1). In contrast, when cultured on Matrigel, ZG cells retained their epithelial-like morphology (Fig. 3B2 and C2). Electron microscopy demonstrated that after a 3 day culture on plastic ZG cells possessed the morphology of freshly-dispersed cells: mitochondria with tubular cristae, smooth endoplasmic reticulum (SER) profiles, lack of rough endoplasmic reticulum (RER) cisternae, and abundant lipid droplets (Figs. 4 and 5A1), whereas after 6 days they showed elongated mitochondria with laminar cristae, few small lipid droplets, no SER profiles and several dense bodies of probable lysosomal nature (Fig. 5B1). After 10 and 12 days of growth on plastic, ZG cells exhibited a dedifferentiated appearance: mitochondria were slender and provided with few laminar cristae and lipid droplets were absent (Fig. 5C1). In contrast, when cultured on Matrigel, ZG cells kept the ultrastructural features of freshly dispersed cells until the 6th day (Fig. 5A2 and B2). At days 10 and 12, mitochondria were elongated with prevalent laminar cristae, but lipid droplets were still abundant (Fig. 5C2).

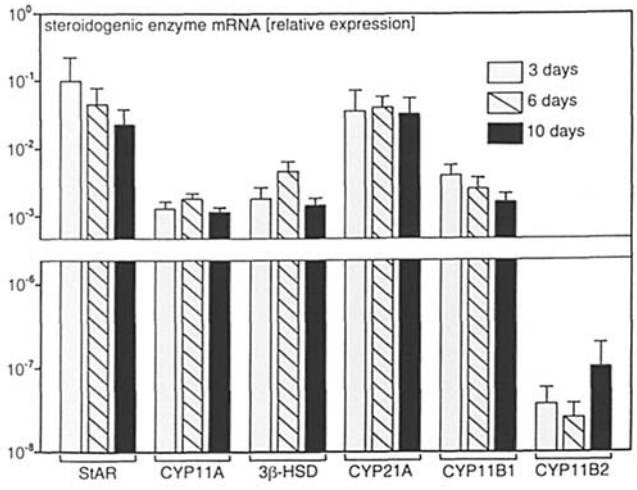

Figure 8. StAR and steroidogenic-enzyme mRNA expression in rat ZG cells cultured on Matrigel. Data are expressed as percent change from the expression in freshly-dispersed ZG cells (taken equal to 1). Bars are means ± SEM (n=4).

Table II. Baseline post-pregnenolone hormone production from dispersed and 3-day cultured rat ZG cells (means ± SEM; n=4).

Hormone (pmol/10^5 cells · h)	Freshly dispersed cells	Cells cultured on plastic	Cells cultured on Matrigel
PROG	125.6±20.8	55.2±9.3	48.3±9.0
DOC	139.8±18.1	72.2±13.4	65.4±11.9
18OH-DOC	98.7±16.5	41.3±8.1	38.2±6.4
CORT	518.5±86.3	270.4±40.1	291.5±42.6
18OH-CORT	386.2±59.7	151.3±26.2	188.2±30.3
ALDO	55.7±8.5	20.2±3.9	18.9±4.1

Steroidogenic-enzyme expression. Real-time PCR evidenced that mRNA expression of StAR and other main steroidogenic enzymes underwent a progressive decrease with the duration of ZG-cell culture on plastic (Figs. 6 and 7). Or the contrary, the culture on Matrigel preserved the expression of all steroidogenic enzymes until day 10 (Figs. 6 and 8). However, it must be noted that the expression of StAR, CYP11A, 3ßHSD, CYP21A and CYP11B1 was from 1 to 3 orders of magnitude less and that of CYP11B2 about 7 orders of magnitude less than in freshly dispersed cells (Fig. 8).

Baseline steroid-hormone secretion. HPLC assay showed that ZG cells cultured for 3 days on both plastic and Matrigel secreted sizeable amounts of post-pregnenolone steroid hormones, but the production was from 48 to 66% less than that of freshly-dispersed cells (Table II). As compared to plastic, Matrigel culture increased the cumulative production of ALDO, 18OH-CORT, CORT and PROG, did not affect 18OH-DOC production, and lowered that of DOC (Fig. 9). The 180-min baseline production of all the steroid hormones assayed decreased with the duration of ZG-cell culture

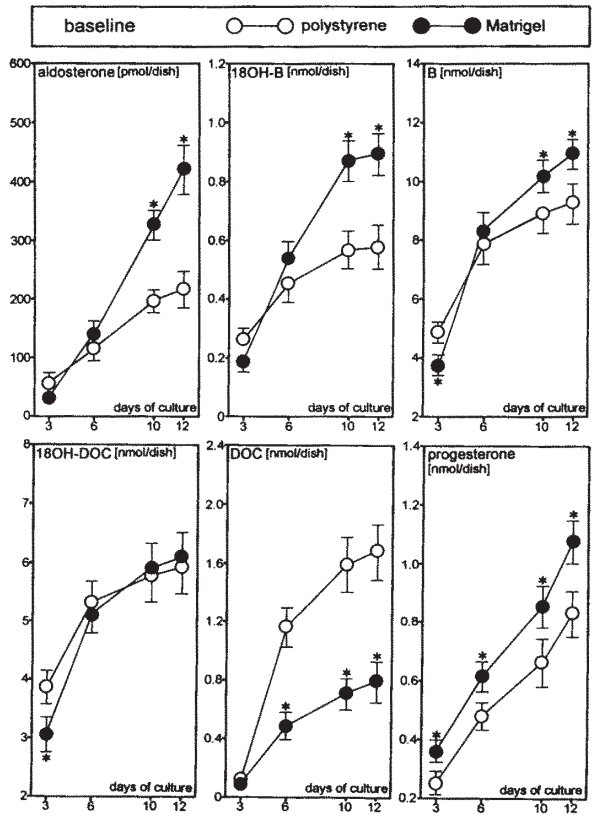

Figure 9. Baseline steroid-hormone cumulative secretion from rat ZG cells cultured on plastic (polystyrene) or Matrigel. Data are means ± SEM (n=4). *P<0.05 from the respective plastic value.

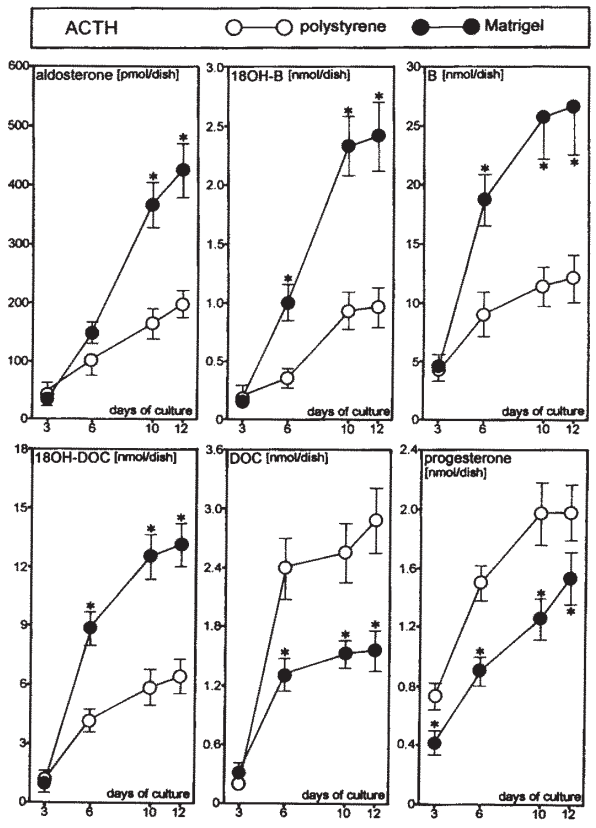

Figure 11. Effect of ACTH on steroid-hormone cumulative secretion from rat ZG cells cultured on plastic (polystyrene) or Matrigel. Data are means ± SEM (n=4). *P<0.05 from the respective plastic value.

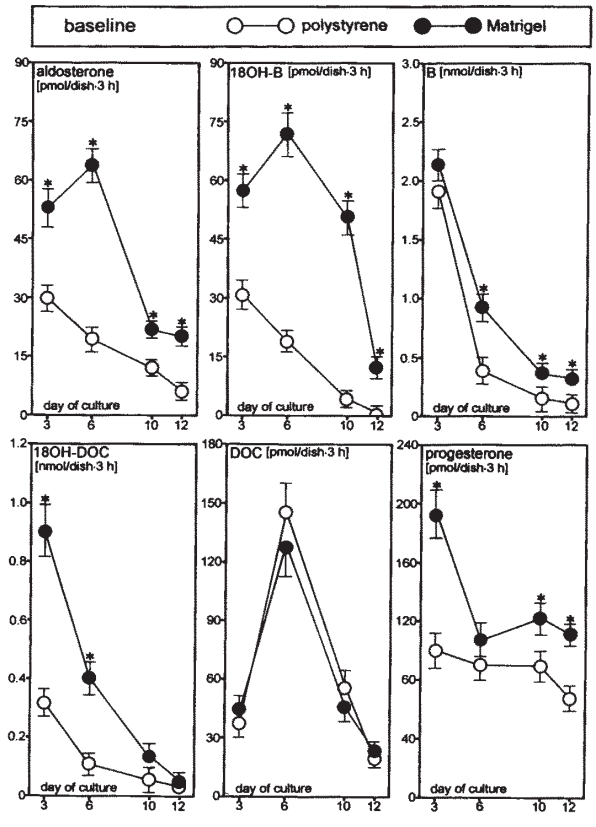

Figure 10. Baseline 180-min steroid-hormone production from rat ZG cells at days 3, 6, 10 and 12 of culture on plastic (polystyrene) or Matrigel. Data are means ± SEM (n=4). *P<0.05 from the respective plastic value.

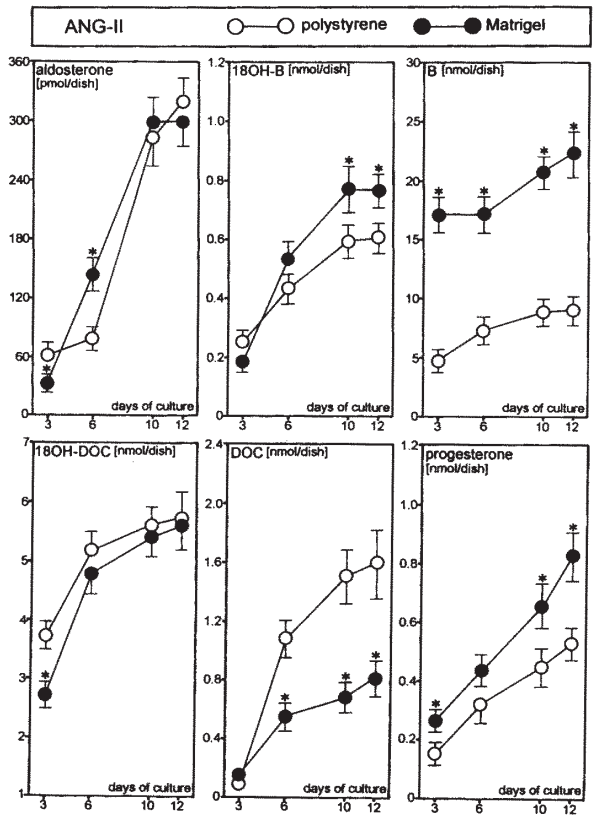

Figure 12. Effect of Ang-II on steroid-hormone cumulative secretion from rat ZG cells cultured on plastic (polystyrene) or Matrigel. Data are means ± SEM (n=4). *P<0.05 from the respective plastic value.

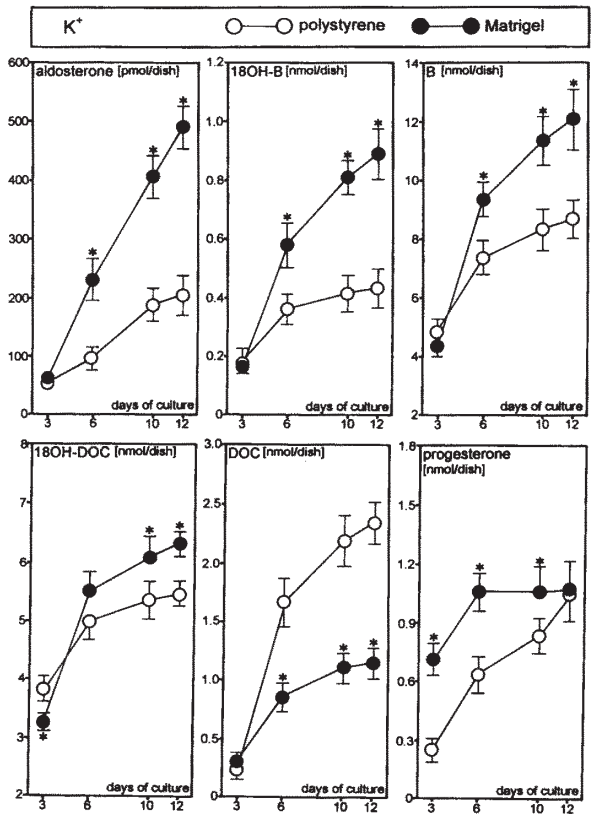

Figure 13. Effect of K^+ on steroid-hormone cumulative secretion from rat ZG cells cultured on plastic (polystyrene) or Matrigel. Data are means ± SEM (n=4). *P<0.05 from the respective plastic value.

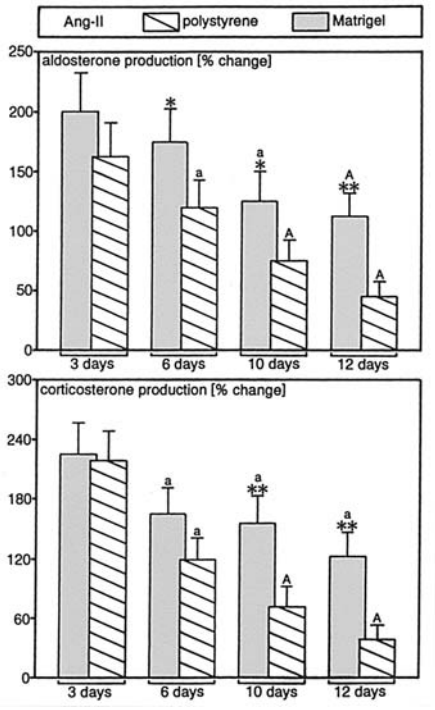

Figure 15. Aldosterone and corticosterone secretory response to Ang-II of rat ZG cells at days 3, 6, 10 and 12 of culture on plastic (polystyrene) or Matrigel. Data are expressed as percent change from the respective baseline value. Bars are means ± SEM (n=4). *P<0.05 and **P<0.01 from the respective plastic value; aP<0.05 and AP<0.01 from the respective 3-day value.

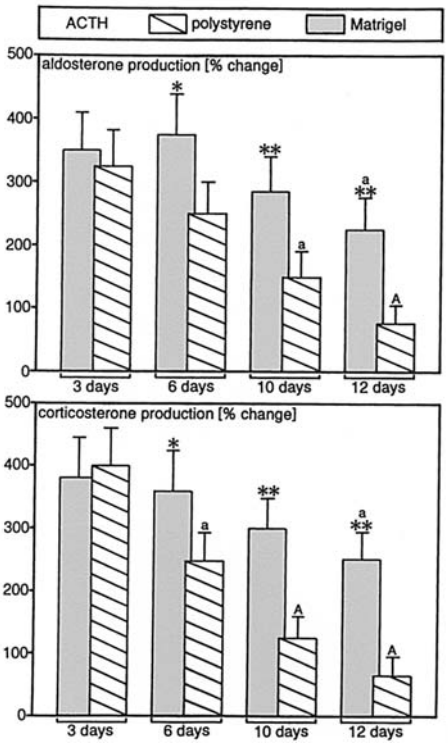

Figure 14. Aldosterone and corticosterone secretory response to ACTH of rat ZG cells at days 3, 6, 10 and 12 of culture on plastic (polystyrene) or Matrigel. Data are expressed as percent change from the respective baseline value. Bars are means ± SEM (n=4). *P<0.05 and **P<0.01 from the respective plastic value; aP<0.05 and AP<0.01 from the respective 3-day value.

(Fig. 10). With the exception of DOC production, this parameter was significantly higher when cells were grown on Matrigel (Fig. 10). Noteworthy is that the 180-min production of ALDO and 18OH-CORT from ZG cells cultured on Matrigel was higher at day 6 than day 3 (Fig. 10).

Agonist-stimulated steroid-hormone secretion. As expected, the repeated exposure to agonists raised the cumulative secretion of post-pregnenolone steroid hormones. Matrigel favored the secretory response (Figs. 11-13), but striking differences were found depending on the agonist. Matrigel: i) enhanced ACTH- and K^+-stimulated cumulative secretion of ALDO, 18OH-CORT, CORT and 18OH-DOC, but lowered that of DOC (Figs. 11 and 13), ii) augmented the Ang-II-stimulated cumulative production of 18OH-CORT and CORT, leaving unchanged that of ALDO and 18OH-DOC and decreasing that of DOC (Fig. 12); and iii) lowered ACTH-stimulated PROG secretion (Fig. 11), but raised the Ang-II- and K^+-stimulated one (Figs. 12 and 13). The acute response to agonists of the two main hormones secreted by rat ZG cells, i.e. ALDO and CORT (22), was assayed by measuring the 180-min hormone production before and after the exposure to agonists and calculating its percent change (Figs. 14-16). The secretory acute response to all agonists decreased with the number of days of culture on plastic. The response to ACTH and Ang-II of ZG cells also decreased with the duration of culture on Matrigel, although it was significantly higher than that of cells grown on plastic (Figs. 14 and 15). Conversely, the secretory response to K^+ did not undergo any change in relation to the number of days of culture on Matrigel (Fig. 16).

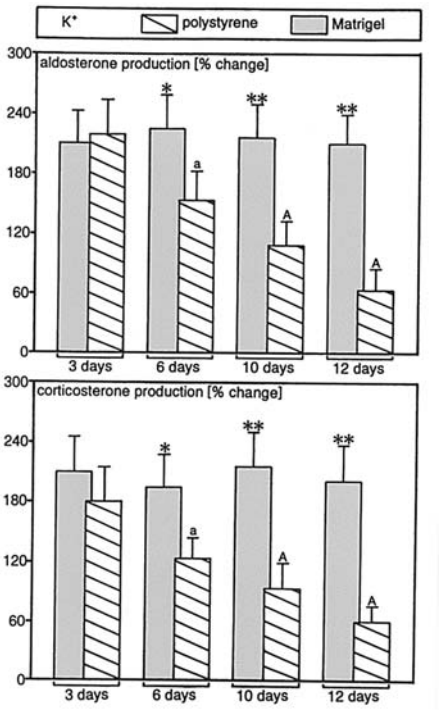

Figure 16. Aldosterone and corticosterone secretory response to K^+ of rat ZG cells at days 3, 6, 10 and 12 of culture on plastic (polystyrene) or Matrigel. Data are expressed as percent change from the respective baseline value. Bars are means ± SEM (n=4). $^{*}P<0.05$ and $^{**}P<0.01$ from the respective plastic value; $^{a}P<0.05$ and $^{A}P<0.01$ from the respective 3-day value.

Discussion

Definitive evidence indicates that steroid-hormone synthesis occurs via a complex pathway involving several enzymes located in both mitochondria and SER. The tubular arrangement of the mitochondria inner membrane, occurring in all steroidogenic cells, is thought to reflect the peculiar content of CYP enzymes (for review see refs. 1,22,23). Briefly, esterified cholesterol, stored in lipid droplets, is trasformed into free cholesterol, that reaches mitochondria, where StAR translocates it to the inner membrane. Here CYP11A transforms cholesterol to pregnenolone, which is released into the cytosol and sequentially converted to PROG and DOC by 3ß-HSD and CYP21A located in SER. DOC enters again into mitochondria, where CYP11B1 11ß-hydroxylates it to CORT. ZG-cell mitochondria also possess CYP11B2, which catalyzes the conversion of DOC to ALDO, via three consecutive reactions: 11ß-hydroxylation of DOC to CORT, 18-hydroxylation of CORT to 18OH-CORT, and finally 18-methyl-oxidation of 18OH-CORT to ALDO. CYP11B2 may also convert DOC to 18OH-DOC, which then is transformed into 18OH-CORT and ALDO (Fig. 1). ZG cells not only secrete CORT and ALDO, but also variable amounts of other post-pregnenolone intermediate hormones (PROG, DOC, 18OH-DOC, and 18OH-CORT), while under normal conditions pregnenolone is not released, because it is quantitatively converted to PROG. In fact, to assay the effect of agonists on the rate-limiting step of steroid synthesis (i.e. StAR/CYP11A-mediated conversion of cholesterol to pregnenolone) is necessary to block 3ß-HSD activity.

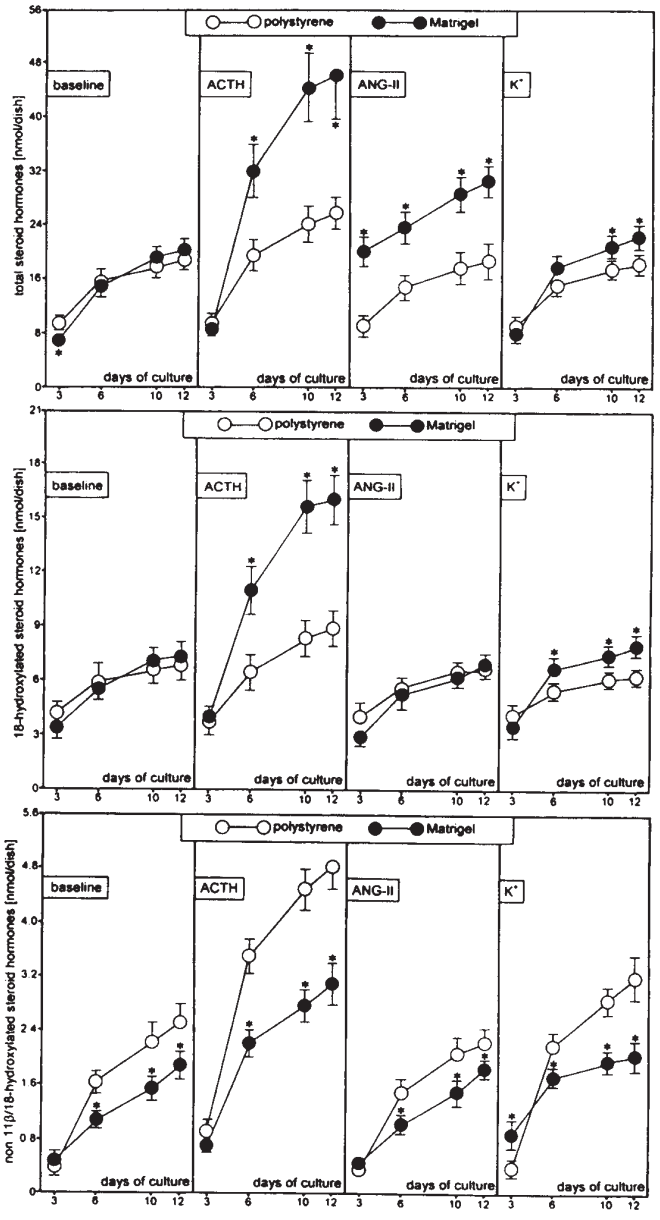

Figure 17. Baseline and agonist-stimulated cumulative secretion of total post-pregnenolone (upper panels), 11ß/18-hydroxylated (middle panels) and non-11ß/18-hydroxylated steroid hormones (lower panels) from rat ZG cells cultured on plastic (polystyrene) or Matrigel. Data are means ± SEM (n=4). $^{*}P<0.05$ from the respective plastic value.

Our present findings clearly show that the culture on Matrigel allows rat ZG cells to retain their well differentiated phenotype for longer periods than the culture on plastic. ZG cells grown on plastic after 6 days display a fibroblast-like appearance and a net lowering in the expression of all steroidogenic enzymes, coupled to a decrease in both basal and agonist-stimulated steroidogenic capacity. In contrast, when cultured on Matrigel ZG cells exhibit the morphological features of actively steroid-secreting cells: mitochondria with tubular cristae, SER profiles and abundant lipid droplets. Moreover, the expression of the steroidogenic enzymes does not undergo appreciable changes and steroid-hormone production, although decreasing in relation to the number of days of stay in culture, remains always higher than that of cells grown on plastic.

The morphological feactures of ZG cells cultured on Matrigel requires further comments, especially as far as lipid droplets and mitochondria are concerned. Lipid droplets are abundant until day 12 of culture, raising the question of the source of esterified cholesterol stored in them. Cholesterol is prevalently taken-up from circulating high-density lipoproteins (HDL), through a receptor-mediated mechanism (scavenger receptor class B type I, SR-BI), or may be locally synthesized from acetate via a series of enzymes located in SER (24-26). Cultured ZG cells may obtain HDL from FCS added to the culture medium, and Matrigel could maintain either SR-BI expression or endogenous cholesterol SER synthesis. At days 10 and 12 of culture on Matrigel, ZG-cell mitochondria display laminar more than tubular cristae, but it is to be noted that this quite dedifferentiated morphology does not prevent mitochondria from participating in steroidogenesis in H-295 cells and adrenocortical-secreting tumors (1,4).

Based on these lines of evidence, it is clear that the culture on Matrigel by preserving the expression of all steroidogenic enzymes favors the production of CORT and ALDO from ZG cells. Less clear is the effect of Matrigel on the production of other post-pregnenolone hormones, if the fact that our HPLC assay gives a dynamic picture of steroid synthesis is not taken into account. A decrease in the early intermediate products (PROG and DOC) may depend on either a lowering in the conversion of cholesterol to pregnenolone or to an enhanced utilization of them in CORT and ALDO synthesis. Hence, it is of interest to analyze the effects of Matrigel on baseline and agonist-stimulated total, 11ß-/18-hydroxylated, and non-11ß-/18-hydroxylated hormone production (Fig. 17). The analysis shows that Matrigel does not affect baseline total hormone cumulative production, but markedly raises the agonist-stimulated one: this depends on the apposite effects of Matrigel on 11ß-/18-hydroxylated and non-11ß-/18-hydroxylated hormone productions, which are either increased (ACTH- and K^+-stimulated ones) or unaffected (baseline and Ang-II-stimulated ones) and decreased, respectively.

Our study also leads to other interesting conclusions: i) the conventional culture on plastic may be satisfactorily used in short-term experiments on rat ZG cells. The beneficial effect of Matrigel on the maintenance of ZG-cell differentiated phenotype becomes evident only starting from day 6 of culture; ii) 6 days of culture on Matrigel are needed for the full recovery of ZG-cell ALDO secretory capacity. The baseline 180-min production of both ALDO and 18OH-CORT is markedly higher at day 6 than at day 3 of growth on Matrigel; and iii) Matrigel culture favors the maintenance of the expression of either ACTH and Ang-II receptors or voltage-gated Ca^{2+} channels in ZG cells. The steroidogenic effects of ACTH and Ang-II are mediated by the MC2 (27,28) and the AT1 receptors (29), while K^+ depolarizes the cell plasma membrane with the ensuing opening of L-type Ca^{2+} channels (30,31). Tissue culture on plastic causes a time-dependent decrease in the secretory response of ZG cells to all their main agonists, while the growth on Matrigel maintains higher the response to ACTH and Ang-II and unchanged that to K^+.

Only speculations are at present possible on the mechanisms underlying Matrigel effects on cultured rat ZG cells. However, evidence has accumulated that extracellular matrix plays an important role in the functional control of adrenocortical cells. Adrenocortical cells are able to produce matrix components (laminin, fibronectin, collagen I and collagen IV) and possess integrin receptors (32-36). Extracellular matrix was found to contribute to the maintenance of 11ß-hydroxylase and c21-hydroxylase activity in cultured bovine adrenocortical cells (37), and collagen IV and fibronectin to enhance 3ß-HSD and CYP17 (17α-hydroxylase) mRNA expression and to maintain the nuclear localization of DAX-1 in cultured human fetal adrenocortical cells (38,39). Finally, the various extracellular matrix components have been shown to variously affect either basal or agonist-stimulated steroid production from cultured adrenocortical cells: collagen IV enhances ACTH- and Ang-II-stimulated cortisol and dehydroepiandrosterone (DHEA) secretion; fibronectin and laminin decrease ACTH-stimulated cortisol, but raise DHEA production, and finally fibronectin, by interacting with integrin receptors, favors ACTH- and Ang-II-stimulated ALDO secretion from rat ZG cells grown on poly-l-lysine coverslips (36,38,40). In this connection, we recall that extracellular matrix has been reported to enhance steroid-hormone production from porcine and ovine ovary granulosa cells (41,42), and to control ACTH secretion from pituitary corticotrophs (43,44). The major components of Matrigel are laminin, collagen IV and heparan sulfate proteoglycans (8), and in light of the above reviewed investigations it appears likely that, by interacting with integrin receptors, Matrigel may favor the long-term maintenance of the differentiated phenotype of rat ZG cells in culture. In view of the specific effect of fibronectin on ALDO secretory capacity of rat ZG cells (36,40), further investigations are under way to examine whether the addiction of fibronectin to Matrigel could improve our culture technique.

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