J. Anat. (1996) **188**, pp. 375–381, with 4 figures Printed in Great Britain

Cell proliferation and renewal of mouse adrenal cortex

YOSKY KATAOKA[1], YUZURU IKEHARA[2] AND TAKANORI HATTORI[2]

[1] *Department of Physiology, Faculty of Medicine, Kyoto University, and* [2] *Department of Pathology, Shiga University of Medical Science, Japan*

(*Accepted 5 September 1995*)

ABSTRACT

Although many hypotheses concerning cell proliferation and renewal in the adrenal cortex of mammals have been proposed, this topic has so far not been elucidated. Adrenocortical cells of adult mammals have low proliferative activity and take a considerable length of time to be renewed. This makes it difficult to investigate the dynamic features of their proliferation. To clarify the cell kinetics, we undertook a long term study in mice using an autoradiographic technique. We radiolabelled almost all the cells throughout the body in newborn mice with the exception of the neurons in central nervous system by the frequent subcutaneous injections of [^{3}H]thymidine every 6 h for 30 d (pulse labelling). After this sequence of pulse labelling, we observed autoradiographically a decrease in the number of ^{3}H-labelled cells in the adrenal cortex as a result of replacement with proliferated unlabelled cells (renewed cells). Single injections of [^{3}H]thymidine (flash labelling) was also performed to examine DNA synthesis in the adrenal cortex. The investigations indicated that the adrenocortical cells proliferate at the border between the zona glomerulosa and the zona fasciculata, and that renewed cells which proliferated in that region move with time bidirectionally towards the cortical surface and the inner (medullary) surface. Half of the cortical cells in the zona glomerulosa, zona fasciculata and zona reticularis were replaced by renewed cells in 30, 60 and 120 d respectively. It took 200 d for almost all cortical cells to be replaced by renewed cells.

Key words: Adrenal cortex; cell proliferation; cell renewal; [^{3}H]thymidine.

INTRODUCTION

The mammalian adrenal cortex consists of 3 laminated regions based on their histological features, namely zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR). It plays an important role in the maintenance of biological homeostasis through the production and release of steroid hormones. Adrenocortical cells produce 3 main groups of steroid hormones, i.e. mineralocorticoids, glucocorticoids and sex steroids. Each group of steroids is produced in particular laminal regions in the cortex. Mineralocorticoids are thought to be produced in the ZG, and glucocorticoids and sex steroids are in the ZF and ZR (Shinzawa et al. 1988; Sasano et al. 1989; Ogishima et al. 1992; Mitani et al. 1994). It remains to be established whether adrenocortical cells which produce different kinds of steroids originate from identical stem cells, and how long cortical cells which produce a particular steroid hormone take to be renewed. Clarification of the cell kinetics of the adrenal cortex is important not only for elucidation of their cell biology but also in relation to pathological and clinical aspects of steroid homeostasis.

So far there have been 4 main hypotheses concerning the cell kinetics of the adrenal cortex (Nussdorfer, 1986). (1) The adrenocortical cells proliferate in ZG and migrate unidirectionally down to ZR through ZF, and eventually die in ZR (Gottschau, 1883; Ford & Young, 1963; Wright et al. 1973). (2) Cortical cells proliferate at the border between ZG and ZF, and migrate bidirectionally towards the cortical surface on the one hand and towards the inner (medullary) surface, on the other (Deane & Greep, 1946; Cain & Harrison, 1950). (3) Cortical cells, which proliferate at the border between ZG and ZF, migrate through ZG along cordonal units forming loops, and then migrate unidirectionally down to ZF–ZR (Nussdorfer, 1980). (4) These cells proliferate in each zone independently (Swann, 1940; Sarason, 1943). Almost all these hypotheses have been based on histological observations on

Correspondence to Dr Y. Kataoka, Department of Physiology, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan.

newborn mammals or under experimental conditions, e.g. pharmacological manipulation, castration or unilateral adrenalectomy, to induce an increase in cell proliferation in the cortex, since adrenocortical cell turnover in adult mammals is slow under normal conditions. Experimental methods under physiological conditions have not been available to provide conclusive evidence as to adrenocortical cell kinetics (Diderholm & Hellman, 1960; Wright, 1971; Zajicek et al. 1986). Under these experimental conditions, however, it has been difficult to follow the cortical cells for prolonged periods in a constant environment after their proliferation, and the experimental findings are considered to be quite different from those obtained in the physiological state. In spite of a number of such studies, little is so far known about the turnover rate of adrenocortical cell kinetics.

The present study, using pulse labelling with [^{3}H]thymidine in mice (Hattori & Fujita, 1976; Ochi et al. 1988) produced firm evidence that adrenocortical cell kinetics extend over more than a hundred days under physiological conditions.

MATERIALS AND METHODS

Flash labelling with [^{3}H]thymidine

Three male ICR mice in each of 2 age groups (3 and 6 months) received a single subcutaneous (s.c.) injection of [^{3}H]thymidine (18.5 kBq/g body weight) and were killed 1 h after the injection.

Pulse labelling with [^{3}H]thymidine

Twenty-four newborn male ICR mice were used. Each received a series of 120 s.c. injections of [^{3}H]thymidine (18.5 kBq/g body weight) every 6 h for 30 d after birth. Three animals were killed at 0, 18, 30, 60, 90, 120, 150 and 200 d after the final injection.

Processing for autoradiography

The mice injected with [^{3}H]thymidine were perfused via the left cardiac ventricle with 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) under the deep anaesthesia with ethyl ether, and both adrenal glands were removed from each animal. The glands were further fixed in the same fixative at 4 °C. After washing in PBS, they were dehydrated through a graded series of ethanol and embedded in paraffin wax. Sections were cut at 4 μm, dipped in Sakura NR-M2 photographic emulsion and exposed for 8 wk at 4 °C. They were then developed in FD-111 and stained with haematoxylin and eosin.

Estimation of labelling index with [^{3}H]thymidine

The adrenal glands from both sides (12 for the flash labelling and 48 for the pulse labelling study) were used. A section containing the centre of the medulla was selected randomly from several sections of each gland and photomicrographs (×400) of the cortex were taken from each selected section.

We accepted cells with round nuclei in the cortex (see Results) as adrenocortical (parenchymal) cells, and counted all radiolabelled and unlabelled adrenocortical cells in the photomicrographs from each cortex. The mean grain count on a radiolabelled nucleus at pulse labelling day (d) 0 was taken as 100%. At each pulse labelling day, nuclei which had a 25% or greater grain count were accepted as radiolabelled.

In each photomicrograph, the ZG was separated into 2 equal laminal subdivisions, ZF into 4 and ZR into 2. For the pulse labelling procedure, the percentage of labelled cortical cells (labelling index) was calculated for each of the 8 laminal subdivisions for each section at d 0, 18, 30, 60, 90, 120, 150 and 200 after the completion of pulse labelling and variations in the index were assessed. The inner laminal subdivision of ZG and the outermost lamina of ZF (G2 and F1 in Fig. 3) were taken as the border between ZG and ZF. In the flash labelling study, the distribution of labelled cortical cells was noted for the mice of both age groups.

RESULTS

Cell nuclei of 2 types were observed in the adrenocortical tissue sections, one being round and the other narrow. All nuclei of both types were labelled with [^{3}H]thymidine immediately after the 30 d series of pulse labelling. As already stated, cells with round nuclei were regarded as adrenocortical cells and the estimations of the labelling index were made on these cells, both in the flash labelling and pulse labelling studies. Cells with narrow nuclei were considered to be fibroblasts or vascular endothelial cells and were excluded.

Flash labelling study

The distribution of DNA synthesising adrenocortical cells was studied in 3 and 6-month-old mice following a single injection of [^{3}H]thymidine. In the 3-month-old mice, 10 to 27 labelled cells were observed in each tissue section, and the labelling index in the whole cortex was 0.23 ± 0.05% (mean ± S.D.; n = 6 cortices);

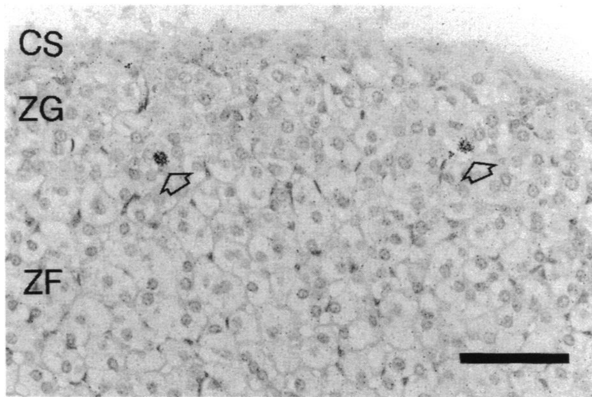

Fig. 1. Flash labelling study in the adrenal cortex of a 3-month-old mouse. Two DNA synthesising cells (^{3}H-labelled cells, arrows) are located at the border between the zona glomerulosa (ZG) and zona fasciculata (ZF). CS, cortical surface (adrenal capsule). Bar, 50 µm.

84 ± 5% (6 cortices) of the labelled cortical cells were located at the border between ZG and ZF (Fig. 1). In the 6-month-old mice, 14 to 23 labelled cells were observed in each section, the labelling index being 0.25 ± 0.08% (6 cortices); 75 ± 10% (6 cortices) of the labelled cells were located at the border between ZG and ZF. In both the 3 and 6-month-old mice, other labelled cortical cells away from the border between ZG and ZF were observed scattered throughout the cortex. These observations indicated that DNA synthesis is most active in adrenocortical cells at the border between ZG and ZF in such adult mice.

Pulse labelling study

By injecting [^{3}H]thymidine at 6 hourly intervals for 30 d, starting immediately after birth, we were able to radiolabel all the DNA synthesising cells throughout the bodies of the mice. These frequent injections of [^{3}H]thymidine produced no detectable histological or other morphological change, including hyperplasia or degeneration, in any laminal zone in the adrenal cortex. All adrenocortical cells were found to be labelled at d 0 (Fig. 2*a*). This indicates that all adrenocortical cells in newborn mice undergo DNA

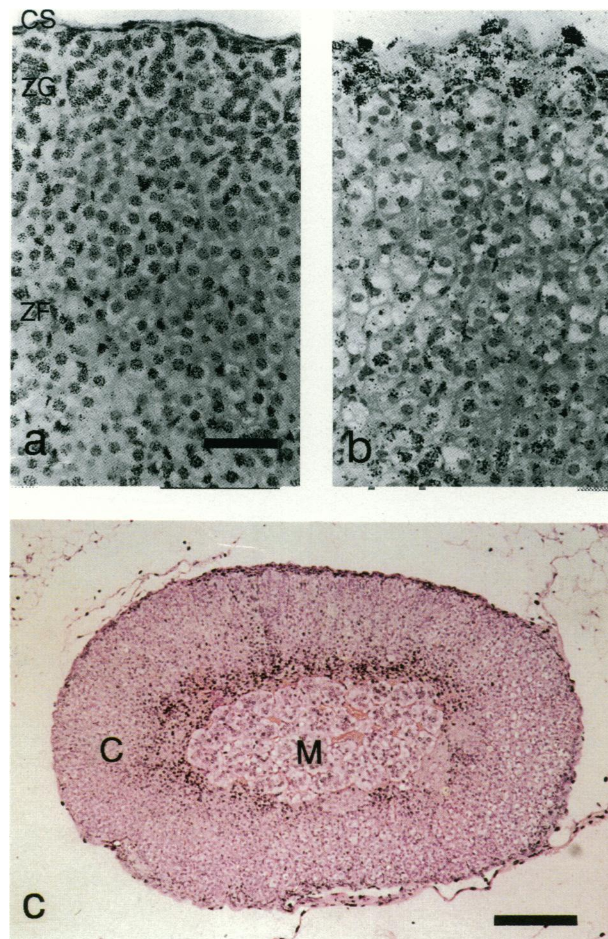

Fig. 2. Pulse labelling study in the adrenal cortex at the d 0 pulse labelling day (*a*), at d 60 (*b*) and at d 120 (*c*). CS, cortical surface; ZG, zona glomerulosa; ZF, zona fasciculata; C, adrenal cortex; M, adrenal medulla. Bars (*a*) 50 µm; (*a*) and (*b*), are at the same magnification; (*c*) 250 µm.

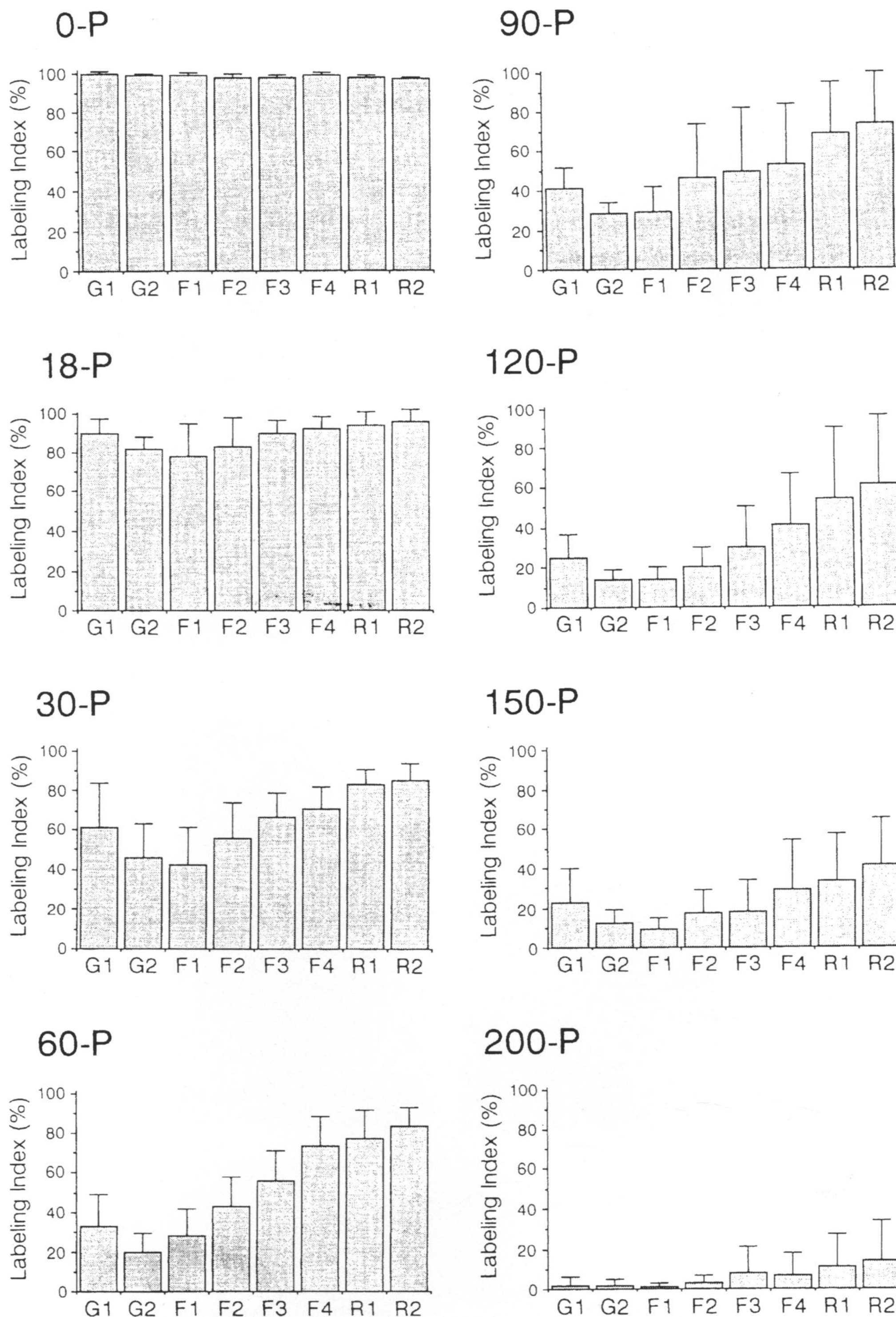

Fig. 3. Percentage of ^{3}H-labelled cells (labelling index) in each laminal subdivision of the cortex (n = 6 adrenal cortices; mean ± S.D.) at each pulse labelling day (0-P to 200-P). The zona glomerulosa, zona fasciculata and zona reticularis are separated into 2 (G1, G2), 4 (F1–F4) and 2 (R1, R2) laminal subdivisions, respectively.

synthesis at least once within 30 d. The labelling index decreased with time, and at any time except d 0, the index at the border between ZG and ZF (at G2 or F1 in Fig. 3) was lower than that at other laminal areas of the cortex. Thus, in mice killed on d 18 unlabelled adrenocortical cells which had proliferated after the period of pulse labelling began to appear at the border between ZG and ZF, and such unlabelled cells then extended with time from the border between ZG and ZF bidirectionally towards the surface of the cortex

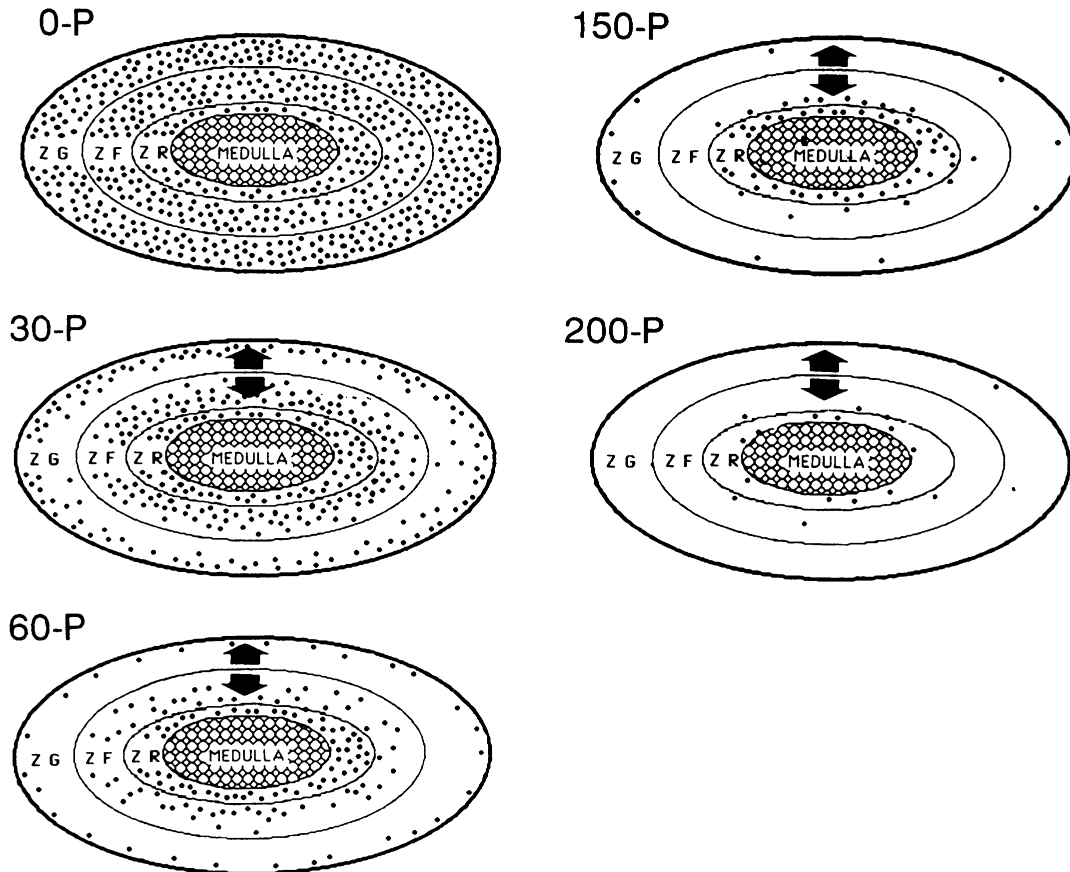

Fig. 4. Diagrammatic representations of the distribution of ^{3}H-labelled cortical cells (dots) at d 0, 30, 60, 150 and 200 pulse labelling days (0-P, 30-P, 60-P, 150-P and 200-P). These diagrams are based on the data in Figure 3. The area including labelled cells began to disappear at the border between the zona glomerulosa (ZG) and zona fasciculata (ZF) and was displaced bidirectionally (arrows) towards the cortical surface and inner medullary surface with time.

and the inner or medullary surface (Fig. 4). Half of the labelled cells in the whole cortex were replaced by unlabelled cells at the d 60 and d 90 time points. Half of the labelled cortical cells were replaced by unlabelled cells at the d 30 stage in ZG, at d 60 in ZF, and d 120 in ZR (Figs 2, 3). Subsequently, 80% of the labelled cortical cells were found to have been replaced at the d 120 stage in ZG, at d 150 in ZF, and at d 150 or d 200 in ZR. Finally, almost all adrenocortical cells throughout the whole cortex became unlabelled by d 200 (Fig. 3).

DISCUSSION

Cell proliferation and renewal in the adrenal cortex

We identified proliferating adrenocortical cells as ^{3}H-labelled cortical cells in the flash labelling study. In addition, we observed evidence of renewal of older cortical cells (^{3}H-labelled cells) by proliferated cortical cells (unlabelled cortical cells) in the pulse labelling study. Adrenocortical cell nuclei which had a grain count of less than 25% of the mean count at the d 0 pulse labelling day were defined as unlabelled. Thus, adrenocortical cells which had divided about 3 times or more after the pulse labelling series were regarded as unlabelled and were taken to represent renewed cells.

These 2 studies produced no detectable histological or other morphological change in any part of the adrenal cortex. This implies that neither the single injection nor frequent injections of tritiated thymidine affected the physiological cell kinetics in the adrenal cortex. Our observations therefore support the 2nd hypothesis listed in the Introduction, that is, most adrenocortical cells of mice proliferate at the border between ZG and ZF, and then migrate bidirectionally, both towards the cortical surface and to the inner medullary surface. In addition, the time taken for cortical cell renewal in each of the 3 laminal zones was clarified. In the adrenal cortex as a whole, it takes 60 to 90 d for half of the cells to be replaced, 150 d for 80%, and 200 d for almost all the cortex to be replaced by renewed cells.

There are previous reports as to the relative numbers of replicating cells in the cortex using tritiated thymidine (Messier & Leblond, 1960) or bromodeoxyuridine (BrdU) (Mitani et al. 1994) injected into adult male rats 1 h before death, in what is referred to as flash labelling studies in this report. The relative numbers of ^{3}H and BrdU-positive cells were 0.44–0.53% and 0.35%, respectively, in those reports, whereas a value of 0.23–0.25% was obtained in the flash labelling study on adult male mice in the present report. Furthermore, Mitani et al. reported that the density of the BrdU-labelled cells at the border between ZG and ZF was 3 or more times higher than that in other laminal zones, and that BrdU-labelled cells migrated into the inner zones after 20 d. However, little is known about adrenocortical cell migration, particularly in ZG, and about the time taken for cortical cell renewal in each laminal zone, since it has been difficult to follow the migrating cortical cells precisely for prolonged periods (over a few hundred days) because of the low proliferative activity in the adrenal cortex.

It has been reported that epithelial cells in the gastric (Hattori & Fujita, 1976) or intestinal mucosa are replaced by renewed cells within a short space of time (24–48 h); on the other hand, exocrine or endocrine cells in the pancreas (Magami, 1990) or glial cells in the central nervous system (Hattori et al. 1989) are replaced at a slow rate (over a few hundred days). The pulse labelling study in this report indicates that adrenocortical cells take a considerable time for renewal, comparable to pancreatic exocrine or endocrine cells or CNS glial cells.

Adrenocortical cell migration and basement membrane

Adrenocortical cells form columnar structures oriented radially in relation to the adrenal medulla (Fawcett, 1994). These columnar structures are clearly identified by PAS staining or antilaminin immunohistochemical staining (data not shown) which visualise basement membrane. The basement membrane is observed as multiple distinct lines running from ZG to ZR in conformity with the columnar arrangement. At ZG, the basement membrane produces rounded structures, and at ZF, linear structures, along which cortical cells form rows. At ZR, however, the linear disposition of the basement membrane becomes disarranged and the rows of cortical cells are less definite. Adrenocortical cells arising at the border between ZG and ZF apparently migrate along the columnar structures bidirectionally towards the cortical and medullary surfaces over the basement membrane as do epithelial cells in mucous membranes.

Adrenocortical cell kinetics and steroid hormone synthesis

Some reports have revealed the location of many kinds of cytochrome P-450s, corticosteroid-synthesising enzymes, in the mammalian adrenal cortex (Shinzawa et al. 1988; Sasano et al. 1989; Ogishima et al. 1992; Mitani et al. 1994). These reports have shown that different kinds of corticosteroid-synthesising enzymes exist in ZG and in ZF–ZR, and that adrenocortical cells in ZG produce mineralocorticoids, whereas those in ZF–ZR produce glucocorticoids and sex steroids. Adrenocortical cells may produce different types of steroids following their migration after proliferation.

In relation to clinical aspects, a long lasting hypersteroidism from Cushing's syndrome caused by a functional adenoma of the adrenal cortex or by corticosteroid therapy, gives rise to atrophy and dysfunction of the normal adrenal cortex in man. In such cases, after removal of the adrenal tumour or withdrawal of steroid administration, atrophic adrenal cortices recover morphologically and functionally over several weeks or months. In these cases, despite the more rapid increase in plasma ACTH levels, full recovery takes a long time; the restoration of intrinsic plasma cortisol levels takes 5 months or more (Graber et al. 1965). The time for proliferation of cortisol producing cells distributing in ZF–ZR in the adrenal cortex may contribute to the period for recovery of plasma cortisol levels. It is also known that the recovery of intrinsic plasma glucocorticoids level takes longer time than that for mineralocorticoids. If the manner of renewal of adrenocortical cells in man is similar to that in mice, apart from the effect of the size of their adrenal glands, these clinical phenomena may be influenced by the particular adrenocortical cell kinetics indicated in this study on mice.

REFERENCES

CAIN AJ, HARRISON RG (1950) Cytological and histochemical variations in the adrenal cortex of the albino rat. *Journal of Anatomy* **84**, 196–226.

DEANE HW, GREEP RO (1946) A morphological and histochemical study of the rat's adrenal cortex after hypophysectomy, with comments on the liver. *American Journal of Anatomy* **79**, 117–145.

DIDERHOLM H, HELLMAN B (1960) The cell migration in the adrenal cortex of rats studied with tritiated thymidine. *Acta Physiologia Scandinavica* **50**, 197–202.

FAWCETT DW (1994) *A Textbook of Histology*, 12th edn., p. 503. New York: Chapman and Hall.

FORD JK, YOUNG RW (1963) Cell proliferation and displacement in the adrenal cortex of young rats injected with tritiated thymidine. *Anatomical Record* **146**, 125–133.

GOTTSCHAU M (1883) Struktur und embryonale Entwicklung der Nebennieren bei Saugethieren. *Archiv für Anatomie und Physiologie* **9**, 412–458.

GRABER AL, NEY RL, NICHOLSON WE, ISLAND DP, LIDDLE GW (1965) Natural history of pituitary-adrenal recovery following long-term suppression with corticosteroids. *Journal of Clinical Endocrinology and Metabolism* **25**, 11–16.

HATTORI T, FUJITA S (1976) Tritiated thymidine autoradiographic study of cell migration and renewal in the pyloric mucosa of golden hamsters. *Cell and Tissue Research* **175**, 49–58.

HATTORI T, HAMADA S, MASAKI T, KUSHIMA R, OHMURA M, KATAOKA Y et al. (1989) Turnover of glial cells in the central nervous system. *Biomedical Research* **10**, Suppl. 3, 355–363.

MAGAMI Y (1990) Cell kinetics study on mouse pancreas [^{3}H]thymidine autoradiographic investigation. *Journal of Tokyo Medical College* **48**, 559–569.

MESSIER B, LEBLOND CP (1960) Cell proliferation and migration as revealed by radioautography after injection of thymidine-H^3 into male rats and mice. *American Journal of Anatomy* **106**, 247–285.

MITANI F, SUZUKI H, HATA J, OGISHIMA T, SHIMADA H, ISHIMURA Y (1994) A novel cell layer without corticosteroid-synthesizing enzymes in rat adrenal cortex: histochemical detection and possible physiological role. *Endocrinology* **135**, 431–438.

NUSSDORFER GG (1980) Cytophysiology of the adrenal zona glomerulosa. *International Review of Cytology* **64**, 307–368.

NUSSDORFER GG (1986) Cytophysiology of the adrenal cortex. *International Review of Cytology* **98**, 319–381.

OCHI M, SAWADA T, KUSUNOKI T, HATTORI T (1988) Morphology and cell dynamics of adipose tissue in hypothalamic obese mice. *American Journal of Physiology* **254**, R740–745.

OGISHIMA T, SUZUKI H, HATA J, MITANI F, ISHIMURA Y (1992) Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-450 11β in rat adrenal cortex: histochemical basis for the functional zonation. *Endocrinology* **130**, 2971–2977.

SARASON EL (1943) Morphologic changes in the rat's adrenal cortex under various experimental conditions. *Archives of Pathology* **35**, 373–390.

SASANO H, MASON JI, SASANO N (1989) Immunohistochemical study of cytochrome P-$450_{17\alpha}$ in human adrenocortical disorders. *Human Pathology* **20**, 113–117.

SHINZAWA K, ISHIBASHI S, MURAKOSHI M, WATANABE K, KOMINAMI S, KAWAHARA A et al. (1988) Relationship between zonal distribution of microsomal cytochrome P-450s (P-$450_{17\alpha,\mathrm{lyase}}$ and P-450_{C21}) and steroidogenic activities in guinea-pig adrenal cortex. *Journal of Endocrinology* **119**, 191–200.

SWANN HG (1940) The pituitary-adrenocortical relationship. *Physiological Reviews* **20**, 493–521.

WRIGHT NA (1971) Cell proliferation in the prepubertal male rat adrenal cortex: an autoradiographic study. *Journal of Endocrinology* **49**, 599–609.

WRIGHT NA, VONCINA D, MORLEY AR (1973) An attempt to demonstrate cell migration from the zona glomerulosa in the prepubertal male rat adrenal cortex. *Journal of Endocrinology* **59**, 451–459.

ZAJICEK G, ARIEL I, ARBER N (1986) The streaming adrenal cortex: direct evidence of the centripetal migration of adrenocytes by estimation of cell turnover rate. *Journal of Endocrinology* **111**, 477–482.