Zone-specific cell proliferation during adrenocortical regeneration after enucleation in rats

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The rat adrenal cortex is composed of concentric layers of cells that include the outer zona glomerulosa and an inner zona fasciculata/reticularis separated by the zona intermedia (27). After adrenal enucleation, with removal of the cortex and medulla leaving the adrenal capsule and adherent cortical cells behind, the cortex regenerates (16, 21). Cortical regeneration restores complete structural and functional zonation in 4–6 wk (6, 16, 35).

The process of regeneration is characterized by both cell differentiation and proliferation (36). By most accounts, cell differentiation precedes proliferation. During the initial response to enucleation, the cortical cells left adherent to the capsule express neither cytochrome P-450 aldosterone synthase (P-450aldo), the marker for glomerulosa cells, nor cytochrome P-450 11β-hydroxylase (P-45011β), the marker for fasciculata cells (11). These observations suggest that enucleation induces the glomerulosa cell to dedifferentiate to the intermediol cell phenotype, cells negative for both P-450aldo and P-45011β (11, 27). Increasing the pool of intermedia cells is consistent with the notion that the zona intermedia provides progenitor cells for regeneration (26). Replacement of fasciculata cells by differentiation from intermediol or glomerulosa cells is in accord with ultrastructural studies showing a transition from glomerulosa-like to fasciculata-like mitochondrial cristae during the initial phase of regeneration (36).

Adrenal regeneration requires a rapid and sustained proliferative response to replace the lost inner fasciculata cells and restore steroidogenic function. Using [3H]-thymidine incorporation, Taki and Nickerson (36) showed increased in vivo labeling of cortical cells between 3 and 7 days after enucleation. These data are consistent with earlier studies showing that mitotic figures are observed in the inner cortex as early as 3 days after enucleation, with cell division occurring throughout the cortex by 18 days (16). Although the time course of the proliferative response has been examined, there are no comprehensive studies that have quantified the proliferative response in a zone-specific manner. Our recent study (10) assessing adrenal proliferation during compensatory adrenal growth employed the use of unbiased stereology and triple-label immunohistochemistry to quantify proliferating glomerulosa and fasciculata cells. These experiments showed that the outer fasciculata is the primary adrenal zone responsible for compensatory growth. A goal of the present study was to use a similar approach to characterize the phenotype of proliferating cells during the course of regeneration.

Optimal adrenal regeneration is dependent on a decrease in plasma corticosterone; regeneration is suppressed if corticosteroids are administered or one adrenal is left intact (7, 11, 18, 21, 33). Our previous study (11) showed that the presence of an intact adrenal altered the normal pattern of cortical cell differentiation that follows enucleation and suppressed regeneration. Interestingly, delayed removal of the intact adrenal weeks after enucleation activates a rapid increase in the weight of the initially suppressed adrenal (21). It is not clear whether this adrenal response is compensatory in nature, because enucleated adrenals can undergo compensatory growth (18) or represents a delayed regenerative response. Because most models of regeneration [e.g., enucleation (16) or transplantation (20)] involve acute trauma to the adrenal cortex, it is possible that mechanical injury to the adrenal by changing local factors, such as inflammatory mediators (16) and innervation (38), can direct the regenerative response. A second goal of the present study was to use the use of unbiased stereology and triple-label immunohistochemistry to quantify proliferating glomerulosa and fasciculata cells. These experiments showed that the outer fasciculata is the primary adrenal zone responsible for compensatory growth. A goal of the present study was to use a similar approach to characterize the phenotype of proliferating cells during the course of regeneration.
study was to determine whether the delayed proliferative response to unilateral enucleation that is initially suppressed by an intact adrenal represents regenerative or compensatory growth. This objective was addressed by comparing the temporal pattern of recovery of cortical cells in a zone-specific manner.

**METHODS**

**Materials**

The following supplies and chemicals were purchased: Vectashield and Antigen Unmasking Solution from Vector Laboratories (Burlingame, CA); Naxcel from Pfizer (New York, NY); pentobarbital sodium (Nembutal) from Abbott Laboratories (North Chicago, IL); corticosterone RIA kits from MP Biomedicals (Irvine, CA); 125I-labeled ACTH from Diasorin (Stillwater, MN); aldosterone RIA kits from Diagnostic Products (Los Angeles, CA); Cy5-labeled donkey antirabbit IgG, AlexaFluor 488-labeled goat antimouse IgG, F(ab’)2 fragment donkey antimonouse IgG, and Cy3-labeled donkey antimouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA); mouse anti-Ki67 antigen from DakoCytomation (Carpinteria, CA); and human ACTH-(1–39) from Peninsula Labs (San Carlos, CA).

**Animals**

Male Sprague-Dawley rats (225–275 g; Charles River, Wilmington, MA) were used in all experiments. The rats received food and water ad libitum. Animals were subjected to a 12:12-h light-dark cycle. Animals were allowed 1 wk to adjust to the facilities before any experiments were conducted. All procedures were approved by the University of Minnesota Animal Care and Use Committee.

**Surgery**

Rats were anesthetized (pentobarbital sodium, 6–7 mg/100 g ip) and underwent left adrenal enucleation [unilateral enucleation (ULE)] and right adrenalectomy (ADX), or left sham surgery (Sham) and right ADX via a dorsal midline incision and bilateral subcostal muscle penetrations. Enucleation consisted of an incision of the adrenal capsule and extrusion and removal of the inner cortex and medulla as described previously (11). Sham involved visualizing but not manipulating the adrenal. After surgery, animals received antibiotic (Naxcel, 2 mg/kg im) and were kept warm until fully ambulatory; they were then returned to the animal care facility.

**Experimental Protocols**

To facilitate comparisons across experiments, Fig. 1 outlines the treatments and timing of the experiments described below.

**Experiment I, time course of regeneration.** To determine the relationship between zone-specific cell proliferation and functional recovery during enucleation-induced regeneration, rats (n = 5–6/group) were sampled in the morning at 2, 5, 10, or 28 days after left ULE and right ADX (ULE group) or left Sham and right ADX (Control group). A prestress blood sample was collected via a tail clip procedure as described previously (38). Twenty minutes after tail clip stress, rats were killed by decapitation and trunk blood was collected. Left adrenals were cleaned of connective tissue and fat, weighed, and frozen for immunohistochemistry. Blood was processed for measurement of plasma corticosterone, aldosterone, and ACTH.

**Experiment II, delayed regeneration.** To assess the growth response in enucleated adrenals that occurs in the presence of an intact adrenal, rats (n = 6/group) underwent left ULE and right ADX (ULE group), left Sham and right ADX (Control group), or left ULE and right Sham (ULE/Sham group) (Fig. 1). At 28 days, rats from each treatment group were decapitated, and adrenals were cleaned and weighed. Other rats from the three treatment groups underwent a second surgery at 28 days. For the ULE and Control groups, the second operation consisted of right Sham, whereas for the ULE/Sham group the second operation consisted of right Sham [delayed Sham (dSham)] or right ADX [delayed ADX (dADX)]. At 28 days after the second surgery (56 days after the initial surgery), rats were decapitated, and adrenals were cleaned, weighed, and frozen for immunohistochemistry. “Delayed” refers to the 28-day interval between enucleation and removal of the contralateral adrenal.

**Experiment III, time course of delayed regeneration.** To determine the time course of the adrenal growth response to delayed removal of the intact adrenal, rats (n = 6/group) underwent left ULE and right Sham. At 28 days, one group of rats was killed (0-day time point) and left adrenals were collected as described for Experiment II. Additional rats underwent a second surgery at 28 days that consisted of right Sham (dSham) or right ADX (dADX). At 2, 5, or 10 days after the second surgery (30, 33, or 38 days after the initial surgery), animals were killed by decapitation and trunk blood was collected.
underwent tail clip stress and were killed by decapitation as in Experiment I.

Immunofluorescence Histochemistry

Staining for Ki67, P-450aldo, and P-45011B was performed using a triple-labeling procedure as described previously (10). Frozen adrenals were sectioned (30 μm), postfixed in Zamboni’s solution and incubated overnight with a mouse anti-Ki67 primary, followed by donkey anti-mouse Cy3 secondary and a blocking antibody [F(ab’2) donkey anti-mouse]. Primary antibodies directed against P-450aldo (rabbit Ab) and P-45011B (mouse Ab), generously supplied by Celso Gomez-Sanchez (University of Mississippi Medical Center) were then applied. After overnight incubation, sections were incubated with secondary antibodies (goat anti-mouse Alexa 488 and donkey anti-mouse). Primary antibodies directed against Ki67 and aldosterone (rabbit Ab) and donkey anti-mouse Cy3 secondary and a blocking antibody [F(ab’2) goat anti-mouse].

Unbiased Stereology and Photomicroscopy

Optical images were collected using a monochrome charge-coupled device camera, captured with a Scion LG-3 frame grabber, and processed on a Macintosh computer using NIH Image software. Triple-labeled images were pseudocolored and overlapped using Adobe Photoshop.

Cell counting was done using unbiased stereology as described by Howard and Reed (19). Cells labeled for Ki67 and P-450aldo or for Ki67 and P-45011B were counted as proliferating glomerulosa cells or fasciculata cells, respectively. Outer fasciculata cells were classified as P-45011B-positive cells adjacent to the zona intermedia; the zona intermedia expresses neither P-450aldo nor P-45011B. Inner fasciculata cells were classified as P-45011B-positive cells adjacent to the medulla (Sham) or the fibrin clot (enucleated). Proper care was taken to ensure that these sampling areas were indeed separate and distinct regions of the fasciculata. Because P-45011B expression in the inner cortex cannot be used to distinguish between fasciculata and reticularis cells, counts of inner fasciculata cells likely include cells in the zona reticularis. Sampling areas within each zone were randomly selected. Labeled cells within an area circumscribed by a two-dimensional box (e.g., 40.1 × 400.5 and 133.5 × 133.5 μm for glomerulosa and fasciculata, respectively) were counted throughout the depth of the section. Using these templates, a sampling area in the glomerulosa extended inward from the adrenal capsule and included 5–10 cell layers or ~1,000 cells; a sampling area in the fasciculata included 15–20 cell layers or ~750 cells either extending inward from the zona intermedia (outer fasciculata) or extending outward from the medulla or fibrin clot (inner fasciculata). Approximately 200 Ki67-labeled (Ki67+) cells from eight distinct sampling areas and four different sections were counted for each adrenal and numerical density (labeled cells per volume) was determined.

Tissue processing did not allow labeling for steroidogenic enzymes at 2 days in experiment I, so cell counts were made in the outer cortex without respect to zone. The border of the sampling area was positioned adjacent to the capsule. Cells of the zona glomerulosa and outer zona fasciculata putatively were included in the outer cortex counts. A magnification of ×200 was used.

Plasma Hormone Measurement

Plasma corticosterone was measured by radioimmunoassay (RIA) with a commercially available kit; the intra-assay and interassay coefficients of variation (CVs) for corticosterone were 7 and 13%, respectively. Plasma aldosterone was measured by RIA using a kit; the intra- and interassay CV for aldosterone were 15.9 and 21.8%, respectively. Plasma ACTH was measured via RIA as described previously (22), using 125I-ACTH as tracer. The intra- and interassay CVs for ACTH were 7.6 and 13.3%, respectively.

Statistics

Differences between treatment groups in adrenal weight and the density of Ki67+ cells within a specific adrenal zone were determined by ANOVA. Differences between groups in plasma hormone values were determined by ANOVA corrected for repeated measures (prestress vs. poststress). Individual means were compared using Newman-Keuls post hoc analysis. When necessary, values were subjected to logarithmic or square root transformation before ANOVA to reduce variance. For all statistical analyses, P < 0.05 was required for statistical significance. Analyses were done using commercially available software (GBSTAT 6.5.6; Dynamic Microsystems, Silver Spring, MD).

RESULTS

Experiment I, Time Course of Regeneration

Plasma hormonal response to enucleation. Hormones were measured to examine the functional capability of the regenerating adrenals. Plasma ACTH increased in response to tail clip stress in all groups except the ULE group at 5 days (Fig. 2). Prestress ACTH was increased in the ULE group compared with the Control group at all time points except 28 days; poststress ACTH was increased at 2, 5, and 10 days, but not at 28 days, in the ULE group relative to the Control group. In the ULE group, there was a decrease in pre- and poststress ACTH between 10 and 28 days, suggesting that steroid negative feedback had recovered by 28 days.

Plasma corticosterone increased in response to tail clip stress in all groups (Fig. 2). Prestress corticosterone did not differ between groups at any time point. Poststress corticosterone was reduced in the ULE group at 2, 5, and 10 days, but not at 28 days, after surgery, suggesting that steroidogenic function in fasciculata cells had recovered by 28 days.

The ratio of plasma corticosterone to the log of plasma aldosterone was calculated as a more sensitive estimate of fasciculata function (39). The poststress ratio was increased over prestress in the Control and ULE groups at all time points (Fig. 2). Similarly to the plasma corticosterone response to stress, the poststress ratio was decreased in the ULE group at days 2, 5, and 10, but not at 28 days, after enucleation; increases in the ratio in the ULE group occurred between 10 and 28 days, reflecting recovery of fasciculata function at this time.

Prestress plasma aldosterone was not measured because of insufficient plasma. Poststress plasma aldosterone was reduced in the ULE group at all time points (Fig. 2); aldosterone increased in the ULE group between 10 and 28 days, suggesting limited recovery of steroidogenic function in glomerulosa cells at 28 days.

Zone-specific cell proliferation in response to enucleation. Triple-label immunostaining showed zone-specific variation in the density of Ki67+ cells in Control and ULE adrenals; examples from each time point, except 2 days after ULE, are shown (Fig. 3). Only one example of a Control adrenal is shown because there was little variation across time. In Control adrenals, Ki67+ cells were increased in the outer fasciculata compared with the glomerulosa and inner fasciculata at all time points. At 2 days after surgery, the intensity of labeling for steroidogenic enzymes in ULE adrenals did not permit accurate determination of cell phenotype. The density of Ki67+ cells measured in areas adjacent to the adrenal capsule, putatively including glomerulosa and outer fasciculata cells, did not differ...
between Control and ULE adrenals at 2 days [77,614 ± 6,122 (Control) vs. 61,094 ± 7,959 (ULE) Ki67(+) cells/mm³ cortical tissue]. In response to ULE, Ki67(+) cells increased in the glomerulosa and inner fasciculata, but not the outer fasciculata, at 5 days compared with Control adrenals. At 10 days, Ki67(+) cells in the glomerulosa returned to Control levels, whereas proliferation in the outer and inner fasciculata remained elevated. Also, proliferation in the outer fasciculata peaked at 10 days and was significantly increased compared with Control levels. At 28 days, Ki67(+) cells remained increased in outer fasciculata of ULE adrenals compared with 28-day Control adrenals; the density of Ki67(+) cells in the outer fasciculata of the 28-day Control adrenals was decreased compared with 10-day Control adrenals. The glomerulosa and inner fasciculata did not differ at 28 days (Fig. 4).

Experiment II, Delayed Regeneration

Body weight increased in all groups between 28 and 56 days, but there were no differences between treatment groups at any time point (data not shown). There was no difference in adrenal weight between rats with one intact adrenal (Control) compared with rats with a regenerating adrenal (ULE/ADX) at 28 or 56 days (Fig. 5). Adrenal weight was reduced in the regenerating left adrenal when the right adrenal remained in situ for 28 or 56 days (ULE/Sham vs. ULE; Fig. 5). After removal of the right adrenal at 28 days, growth of the initially suppressed adrenal was observed, as reflected by increased adrenal weight at 56 days (ULE/dADX vs. ULE/dSham; Fig. 5). However, adrenal weight after dADX was reduced compared with adrenal weight in rats that had undergone ULE and immediate ADX (ULE/dADX vs. ULE at 56 days; Fig. 5). These results were unaffected if the adrenal weight was normalized to body weight (data not shown).

Experiment III, Time Course of Delayed Regeneration

There was no difference in body weight between groups at any time point (Table 1). At 2 and 5 days after dADX (ULE/dADX group) there were no differences in adrenal weight, whereas adrenal weight increased at 10 days (Table 1). Prestress plasma ACTH was not elevated after dADX and did not change over time. Poststress ACTH was increased relative to prestress values at all time points in the dADX group. Prestress plasma corticosterone did not vary over time after dADX. Poststress corticosterone did not increase over prestress values at 2 and 5 days, and poststress corticosterone values at 2 and 5 days were significantly lower than ULE/dSham. At 10
days, poststress corticosterone in the dADX group was elevated over prestress values, and poststress corticosterone no longer differed from Shams, suggesting some recovery of fasciculata function (Table 1). Plasma corticosterone in the ULE/dSham group increased in response to stress at all time points; data are included for comparison with the assumption that secretion from the intact adrenal is the major source of corticosterone in this group.

Immunostaining showed zone-specific variation in the density of Ki67$^+$ cells in the ULE/dSham and ULE/dADX adrenals (Figs. 6 and 7). In response to dADX, Ki67$^+$ increased in

![Fig. 4. Adrenal zone-specific cell proliferation at 5, 10, and 28 days after ULE or Sham estimated by the density of Ki67$^+$ cells; $n = 5–6/group$. $^\wedge P < 0.05$ vs. Ctrl; $^# P < 0.05$ vs. preceding time point.](image)

![Fig. 5. Adrenal weight after ULE, Sham (Control), ULE and 28-day delayed ADX (ULE/Delayed ADX), or ULE and delayed Sham (ULE/Sham). Values are means $\pm$ SE; $n = 6$ rats/group. $^* P < 0.05$ vs. ULE; $^\wedge P < 0.05$ vs. ULE/Delayed Sham.](image)
the outer fasciculata at 5 days. By 10 days, Ki67\(^+\) cells in the outer fasciculata continued to increase (see example in Fig. 6); in addition, the density of Ki67\(^+\) cells increased in the inner fasciculata (Fig. 7).

**DISCUSSION**

The primary goal of the present study was to identify the phenotype of the cortical cell that proliferates during regeneration induced by adrenal enucleation. Because regeneration requires both cell differentiation and proliferation (36), characterizing zone-specific proliferation must consider both processes. Enucleation removes the medulla and the inner cortical cells, leaving cells adherent to the adrenal capsule as the source for regeneration. Using in situ hybridization, we have shown previously that the initial response to enucleation is a decrease in the expression of P-450aldo and P-45011\(\beta\) mRNA, reflecting the loss of glomerulosa and fasciculata cells, respectively (11). Although the loss of P-45011\(\beta\) mRNA results from the removal of fasciculata cells, the loss of P-450aldo mRNA is due in part to the dedifferentiation of glomerulosa cells to intermediate cells, cells that express neither P-450aldo nor P-45011\(\beta\) mRNA (11, 27). In the present study, at 2 days after enucleation, the low intensity of labeling for P-450aldo and P-45011\(\beta\) protein precluded identification of cell phenotype. Although Ki67 labeling was observed throughout the cortical remnant, demonstrating high proliferative activity, the phenotype of the regenerating cells could not be ascertained. By 5 days, triple-label immunohistochemistry showed increased proliferation in the glomerulosa and inner fasciculata in enucleated adrenals compared with Sham adrenals. Because Sham adrenals undergo compensatory adrenal growth reflected by increased proliferation in the outer fasciculata at 5 days (10), proliferation in this cortical area was not elevated relative to Sham adrenals. However, by 10 days and continuing through 28 days, the pattern of proliferation changed such that high proliferative activity occurred predominantly in the outer fasciculata. These results suggest that the initial proliferative response to enucleation includes all cortical zones but that subsequent expansion of cortical mass occurs via proliferation predominantly in the outer fasciculata.

The finding that increased cell proliferation occurs concomitantly in the glomerulosa and fasciculata during the early stages of regeneration differs markedly from that observed during other forms of adrenal growth. In the intact adrenal, zone-specific proliferation may represent a homeostatic re-
sponse to a requirement to produce the specific steroidogenic product of that zone. For example, glomerulosa cell proliferation increases in parallel with increased aldosterone secretion in response to a low-sodium diet (24), whereas acute stimulation of fasciculata cell proliferation and corticosterone secretion occurs preferentially after ACTH treatment (28). After unilateral adrenalectomy, compensatory growth of the remaining adrenal occurs via proliferation that is restricted to the outer fasciculata (10). In addition, the surgical stress required for adrenal exposure induces a transient increase in proliferation of outer fasciculata cells (10). As indicated earlier in this section, the high density of Ki67+ cells in the outer fasciculata in the Sham group at 2 and 5 days in the present study likely results from stimuli produced by contralateral adrenalectomy and surgical stress. It appears that the adaptive response to enucleation with concomitant unilateral adrenalectomy initially induces proliferation of all cortical phenotypes. Restoration of fasciculata tissue occurs rapidly, such that plasma ACTH and corticosterone responses to tail clip stress are normalized by 28 days. Calculation of the ratio of plasma corticosterone to the log of plasma ACTH was done as an indirect index of fasciculata responsiveness to ACTH (39); recovery of the poststress ratio occurred in the ULE group over time, reaching a maximum at 28 days that was not different from that of the Control group. These data are consistent with our earlier studies, showing that the rate and extent of recovery of expression of steroidogenic enzymes (11) and steroidogenic function (38) are greater in the fasciculata compared with the glomerulosa. The temporal differences in functional recovery are consistent with the finding that proliferation in the outer fasciculata is elevated at 10 and 28 days, suggesting that addition of cells to the outer fasciculata to expand the zona fasciculata is the predominant regenerative response during this period. The addition of fasciculata cell mass correlates well with previous studies showing the recovery of P-45011β mRNA levels between 2 and 3 wk after enucleation in rats (8, 11).

During the initial phase of regeneration (1–5 days) there is a loss of glomerulosa cells, which is due in part to dedifferentiation reflected by a transition from glomerulosa-like to fasciculata-like mitochondrial cristae (36), by a decrease in expression of P-450aldo mRNA (11), and a suppression of Pref-1, a glomerulosa-specific factor proposed as a mediator of zonal differentiation (17). We (11) and others (17) have hypothesized that glomerulosa cells dedifferentiate to the intermedia cell phenotype, cells that could serve as a progenitor for regeneration of fasciculata cells (26). Although the initial regenerative response may require a pool of progenitor cells, our finding that proliferation of P-45011β-positive cells is elevated from 5 to 28 days after enucleation suggests that expansion of the inner cortical zones proceeds via replication of these cells during this stage of regeneration. Because P-45011β expression in the inner cortex cannot be used to distinguish between fasciculata and reticularis cells, a clear assessment of the time for recovery of the zona reticularis was not possible.

In addition to fasciculata cells, glomerulosa cells demonstrated increased proliferation at 5 days. This response might be important for increasing the initial pool of progenitor cells, but it was not maintained at 10 or 28 days. It is possible that the transient increase in proliferation results, in part, from stimulation by angiotensin II, a primary stimulus for in vivo growth of glomerulosa cells in rats (25). Plasma angiotensin has not been measured after enucleation, but increased sodium retention occurs during the first week of regeneration (14). Enhanced sodium retention is a critical element in the development of adrenal regeneration hypertension after enucleation, which is exacerbated by unilateral nephrectomy and increased salt intake (34). In this model, plasma renin activity increases at 2 days after enucleation, returning to baseline by 10 days (29). Because plasma aldosterone is reduced, increased sodium retention has been attributed to another mineralocorticoid secreted by the enucleated adrenal (31); candidates include deoxycorticosterone (DOC) (3) and a metabolite, 19-noroxycorticosterone (15). Elevated secretion of DOC, the substrate for P-45011β, results from reduced P-45011β activity in regenerating fasciculata cells (2). On the basis of this scenario, restoration of glomerulosa structure and function should proceed when suppression of the renin-angiotensin system by increased sodium retention has abated; presumably, this would occur when P-45011β activity in fasciculata cells returns to normal. A similar sequence of events might have occurred in the present study, although rats did not undergo unilateral nephrectomy or salt loading. Because neither plasma sodium nor sodium excretion were measured in our studies, it is not known whether changes in sodium retention are associated with the recovery of glomerulosa structure and function. Clearly, the plasma aldosterone response to stress is still reduced by 50% at 28 days after enucleation, demonstrating that neither proliferation nor differentiation of glomerulosa cells is sufficient at this time for complete functional recovery of this zone. Additional experiments are required to determine whether glomerulosa function is ultimately restored either via cell proliferation or differentiation. It would also be of interest to examine whether or not changes in sodium balance and renin-angiotensin activity are associated with this recovery.

Optimal regeneration of the adrenal cortex appears to be dependent on a hypocorticoid signal. Enucleation-induced regeneration is suppressed by injections of corticosteroids (33) or by secretion of endogenous steroids from an intact adrenal left in situ (18, 20). Our previous study (11) showed that the presence of an intact adrenal suppressed regeneration, which was reflected by impaired recovery of P-45011β mRNA expression and adrenal weight. Because removal of the intact adrenal weeks after enucleation results in an increase in the weight of the initially suppressed adrenal (21), experiments were performed to examine the proliferative response of enucleated adrenals to delayed adrenalectomy. Our initial experiment (Experiment II, delayed regeneration) showed that adrenal weight at 28 days after enucleation was suppressed if the contralateral adrenal was left in situ and that delayed removal of the contralateral adrenal resulted in increased weight in the enucleated adrenal at 28 days after adrenalectomy. These data confirm the finding that the enucleated adrenal suppressed for 4 wk by the presence of an intact adrenal has the capacity to mount a growth response. To understand the cellular process underlying the adrenal growth, zone-specific proliferation was assessed during the initial 10 days after delayed contralateral adrenalectomy (Experiment III, time course of delayed regeneration). Initial increases in Ki67+ cells were observed in the outer fasciculata at 5 days, and by 10 days increases in proliferation occurred both in the outer and inner fasciculata. The cellular response was paralleled by increased adrenal weight and corticosterone responses to tail clip stress at 10
days, suggesting that functional recovery of the fasciculata was occurring after delayed adrenalectomy. In contrast to the response to enucleation with acute contralateral adrenalectomy (Experiment I, time course of regeneration), prestress plasma ACTH was not elevated after delayed adrenalectomy. These results suggest that the enucleated adrenal secreted sufficient corticosterone by 2 days to prevent the hypocorticoid signal that results in hypersecretion of ACTH (11). Because increased ACTH contributes to the initial regenerative response to enucleation (11), it is likely that delayed proliferative responses in this model of adrenal growth results in part from the lack of an ACTH drive. The initial proliferative response to delayed adrenalectomy most likely represents compensatory growth of the enucleated adrenal, since compensatory growth is characterized by increased proliferation in the outer fasciculata (10). These data are consistent with previous work showing that enucleated adrenals can undergo a compensatory response (18). However, there are clear differences between the delayed regenerative response and compensatory growth. The compensatory response occurs earlier, with peak proliferation at 2 days, is in decline by 5 days, and does not involve the inner fasciculata to a large extent at any time (10). The observation that proliferation also increased in the inner fasciculata at 10 days suggests that the delayed growth of the enucleated adrenal results in part from a regenerative response. However, there are also clear differences between the delayed regenerative response and the immediate response to enucleation, in that proliferation is delayed and does not reach levels comparable to those observed during the immediate response (compare Figs. 4 and 7).

The finding that enucleated adrenals can undergo a regenerative response after chronic suppression is a novel finding with biological and clinical significance. First, the data show that regenerative growth reflected by proliferation in the inner fasciculata can occur without direct injury to the adrenal. Because the proliferative response is delayed compared with enucleation and acute contralateral adrenalectomy, it is likely that stimuli induced by acute adrenal injury do affect the rate and extent of adrenal regeneration. Both the local inflammatory response (30) and disruption of adrenal innervation (38) could affect the response. Second, these experiments add new information showing that limited exposure to glucocorticoids at the time of adrenal injury does not permanently disrupt the capacity to mount a regenerative response. These results are relevant to the clinical situation in which patients are administered glucocorticoids chronically after acute adrenal injury. When adrenal insufficiency is suspected, patients are treated with glucocorticoids (9, 23, 32). Although glucocorticoid therapy might be essential for improving hemodynamic stability and reducing mortality, there has been little attention given to the consequences of glucocorticoid treatment on functional recovery of the damaged adrenal. The present study shows that the injured rodent adrenal can be suppressed for 4 wk and yet remain responsive to a growth stimulus. A similar phenomenon can most likely occur in human adrenals, as indicated by the complete recovery of steroidogenic function after severe adrenal insufficiency produced by massive bilateral adrenal hemorrhage (13): although glucocorticoids were given for 10 mo, adrenal mass and function had recovered after 2.5 yr. This observation is a clear example that injured human adrenal tissue has the potential for recovery of steroidogenic function after cessation of glucocorticoid treatment.

Adrenal regeneration requires pituitary secretion of proopiomelanocortin (POMC)-derived peptides because hypophysectomy prevents the response (21). Enucleation-induced reduction in circulating corticosterone results in increased pituitary secretion of ACTH and other POMC-derived peptides (1, 7, 11, 12). Fasciculata cell proliferation could result directly from ACTH (11, 28) or from mitogens produced locally in the adrenal by a secretory protease that has been implicated in compensatory adrenal growth (4, 5). In addition to a direct mitogenic effect, ACTH most likely contributes to the angiogenic response that is required to support the restoration of adrenal parenchyma (37). The delayed-regeneration experiments show that the rate and extent of recovery of tissue mass and function are limited when a hypocorticoid signal is absent or delayed relative to adrenal injury. These findings provide additional support for a prominent role for POMC-derived factors in the timing and course of adrenal regeneration.

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