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Adrenocortical cell transplantation in *scid* mice: the role of the host animals' adrenal glands☆

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Abstract

Adrenocortical cell transplantation is a powerful technique for the investigation of the regulation of adrenocortical structure and function. Some classical organ and tissue transplantation experiments suggest that the success of transplantation depends on the activity of the pituitary gland and other endocrine systems, and is therefore influenced by the host animals' own adrenal glands. For this reason, our experiments have usually been performed on adrenalectomized animals. However, we show here that cell transplantation experiments, involving the introduction of bovine adrenocortical cells into *scid* mice, do produce transplant tissues in the presence of the host animals' adrenal glands. However, the tissue that forms is small and its cells also smaller than usual. When the adrenals of such animals are removed in a second surgical procedure, the transplants show a rapid increase in steroidogenic function and a slower increase in size, over several weeks. We conclude that the initial process by which transplanted adrenocortical cells organize into a tissue structure is not affected by the presence of the host animals' adrenal glands, but the growth of the transplants is limited until the adrenal glands are removed.

Keywords: Transplantation; Adrenal cortex; Steroidogenesis; Proliferation; p21

1. Introduction

Over the past few years, we have developed a new model system for the investigation of adrenocortical cell biology. In this model, bovine or human adrenocortical cells are transplanted into immunodeficient (*scid*) mice [1]. When placed in a suitable site in the host animal, the cells form a new functional tissue structure that is vascularized and united with the host vascular system. Moreover, this tissue can replace the essential steroidogenic functions of the animals' own adrenal glands [1]. We have used this model system in several investigations of adrenocortical biology. These experiments have included studies on normal primary adrenocortical cells isolated both from bovine adrenal glands [2,3] and from human adrenal glands from donors of various ages [4,5]. Additionally, we have performed a series of transplantation studies using adrenocortical cells that have been genetically modified, and we have proposed that this cell transplantation model provides a new method for the investigation of gene action, as an alternative to conventional methods [6,7].

The process by which a loose suspension of cells reorganizes into a vascularized tissue, in cooperation with host cells, is not well established and is the subject of intensive investigation in this laboratory [1]. One of the critical questions is the dependence of the growth and function of adrenocortical cell transplants on the endocrine status of the host animal. In this regard, the most obvious factor is the activity of the host pituitary gland, which together with the renin/angiotensin system are the endocrine systems that control the adrenal cortex. The question of the role of the pituitary gland arises particularly in view of older literature in which adrenocortical tissue or organs were transplanted. These experiments were performed within the same species, whereas our experiments are examples of xenotransplantation. The data in the older literature are conflicting, but there are many studies suggesting that the survival, growth, and function of transplanted adrenocortical tissue is dependent on stimulated pituitary function (presumably involving increased secretion of ACTH, although this was not clearly determined) [8]. At least in some studies, adrenocortical tissue transplanted in animals with intact adrenal glands never became vascularized and functional; the tissue died and was replaced with scar tissue [8].

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With this older data in mind, when we first began work on adrenocortical cell transplantation, our standard protocol was to adrenalectomize the animal at the time of transplantation, so as to remove the inhibitory influence of adrenocortical steroids on pituitary function. Given the success of this protocol, we performed almost all experiments in animals that were adrenalectomized at the time of transplantation. However, the question remains as to whether this assumption was correct. Does the presence of the animals' adrenal glands prevent the proper formation of transplant tissue, or affect the growth and function of the tissue? In order to study this question, we performed a series of experiments in which the animals' adrenal glands were not removed at the time of transplantation. Additionally, we performed experiments in which the animals' adrenal glands were removed several days or weeks following cell transplantation; following this delayed adrenalectomy the animals were allowed to survive from 1 day to several weeks further before sacrifice.

2. Materials and methods

2.1. Growth of bovine adrenocortical cells in culture

Bovine adrenocortical cells were derived by enzymatic and mechanical dispersion from the adrenal cortex of 2-year-old steers, as previously described [9]. Primary cell suspensions were stored frozen in liquid nitrogen. Frozen cells were thawed and replated in Dulbecco's modified Eagle's medium/Ham's F-12 (1/1) with 10% fetal bovine serum, 10% horse serum and 0.1 ng/ml recombinant FGF-2 (Mallinckrodt, St. Louis, MO) [9]. Cells were grown in culture for 7 days before transplantation.

2.2. FGF-transfected 3T3 cells

As previously described, adrenocortical cells were co-transplanted with 3T3 cells stably expressing FGF-1 fused in frame with a signal peptide from hst/KS3, yielding a highly angiogenic secreted product [10]. 3T3 cells were grown under the same conditions as bovine adrenocortical cells. To render the cells incapable of further division after transplantation, they were lethally irradiated, as previously described [3].

2.3. Transplantation of cells beneath the kidney capsule of scid mice

ICR *scid* mice were maintained in an animal barrier facility as a breeding colony. Animals (both males and females) at an age greater than 6 weeks (~25 g body weight) were used in these experiments. Procedures were approved by the Institutional Animal Care Committee and were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The surgical procedure for transplantation of adrenocortical cells beneath the kidney capsule has been previously described [3]. In the standard protocol, mice were adrenalectomized and received transplants of adrenocortical cells in a single procedure.

As in previous experiments, we used a small polycarbonate cylinder to create a virtual space beneath the capsule into which the cells could be introduced [2,3]. Adrenocortical cells were introduced into this space as follows. Cells were released from the culture dish by digestion with bacterial protease [9]. Adrenocortical cells (2×10^6) mixed with 4×10^5 irradiated 3T3 cells were injected into the subcapsular cylinder by a transrenal injection using a 50 μl Hamilton syringe with a blunt 22-gauge needle.

Post-operative care for the animals consisted of the administration of synthetic steroids for 7 days (for animals that were adrenalectomized at the time of cell transplantation) and the administration of analgesics and antibiotics in the drinking water (for all animals). These treatments were described previously [3].

After 2 weeks, and at approximately weekly intervals thereafter, tail blood samples were taken 15 min after the injection of ACTH (Sigma, 0.01 units/g body weight). In the standard protocol, animals were sacrificed 35 days after cell transplantation. Animals were injected with ACTH 15 min before sacrifice and cardiac blood samples were removed under anesthesia.

In some experiments, the adrenal glands were removed in a second surgical procedure. The animals received the standard post-operative care, as described earlier, without steroid administration.

2.4. Histology and immunohistochemistry

The fixation, paraffin embedding and histological examination of tissue formed from transplanted cells were performed using standard techniques. Some tissue sections were stained for expression of the Ki-67 proliferation-associated antigen and for $p21^{WAF1/CIP1/SDI1}$ as previously described [11]. Sections were lightly counterstained with hematoxylin.

Transplants were distinguished from surrounding host tissues by a immunohistochemical reaction with an monoclonal antibody that recognizes an unknown 65 kDa human mitochondrial protein (Chemicon International, Temecula, CA) [12] (see Section 3). Sections were incubated in 1:10 dilution of the antibody. The secondary antibody used was Vector Laboratories' (Burlingame, CA) universal biotinylated secondary antibody.

2.5. Radioimmunoassays

Radioimmunoassays were performed directly on plasma samples from animals with transplanted cells using the following kits. Cortisol: Corti-cote (ICN Pharmaceuticals, Orangeburg, NY). The manufacturer reports that this antibody has a cross-reaction of 1.2% with corticosterone. Corticosterone: ImmuChem double antibody kit (ICN). This antibody has a reported cross-reaction of 0.05% with

cortisol. Aldosterone: Active Aldosterone kit (Diagnostic Systems Laboratories, Webster, TX). The manufacturer reports a cross-reaction of this antibody with corticosterone of 0.03% and no detectable cross-reaction with cortisol.

3. Results

Using the protocol for adrenocortical cell transplantation in *scid* mice that we have developed and previously described, we transplanted primary bovine adrenocortical cells beneath the kidney capsule. The variation from our standard protocol was that some animals were not adrenalectomized at the time of cell transplantation. In adrenalectomized animals, transplants are mature at 35 days, i.e. the tissue is well formed and vascularized and the plasma steroid levels have plateaued [3]. In a series of animals, we transplanted bovine adrenocortical cells without removing the adrenal glands at the time of transplantation and subsequently performed adrenalectomy at 35 days. Animals were then sacrificed at various times ranging from 12 h to 42 days later (Figs. 1–3). In a few animals, adrenalectomy was performed at 7 or 21 days following cell transplantation.

When non-adrenalectomized animals were sacrificed at 35 days, a small transplant tissue was present above the mouse kidney at the site of injection of the cells (Fig. 1). Transplants in non-adrenalectomized animals were substantially smaller than in animals adrenalectomized at the time of cell transplantation [3]. The cells within the tissue in non-adrenalectomized animals were also smaller. In order to ensure that these cells were in fact adrenocortical cells, and not of host origin, we used an immunohistochemical technique to distinguish adrenocortical cells from surrounding host tissue. An antibody against a mitochondrial protein was found to stain bovine adrenocortical cells without any reaction with host mouse tissue. This antibody was previously reported to be useful in distinguishing transplanted human cells in the mouse brain [12], and we also found in other experiments that it intensely stains human adrenocortical cells in transplants. As it reacts with human and bovine cells of various types, but does not react with mouse cells, it is assumed that the protein differs significantly among these species. Although the identity of the protein is unknown, this antibody technique has proved to be very useful in distinguishing transplanted cells from host cells, particularly in distinguishing scar tissue, which is quite frequently seen in parts of the transplants. Using this technique, it is evident that the transplants in non-adrenalectomized animals, although smaller, are indeed composed of bovine adrenocortical cells (Fig. 1).

Over a period of weeks, transplants in animals following delayed adrenalectomy showed a normalization of the size of the tissue and of cell size (Fig. 1). In the first week following adrenalectomy, the structure of the transplants showed few visible changes (an example is shown in Fig. 1 of an animal sacrificed 24 h after adrenalectomy). By 42 days following adrenalectomy, transplants had increased substantially in size, and the cells had also enlarged. Two examples are shown in Fig. 1.

In view of the difference in the size of transplant tissues in non-adrenalectomized animals, we investigated cell proliferation in the transplants, assessed by immunohistochemical detection of the proliferation marker Ki-67. We previously noted that the monoclonal antibody used does not stain dividing host mouse cells. Fig. 2 shows dividing cells in a control transplant (animals adrenalectomized at the time of cell

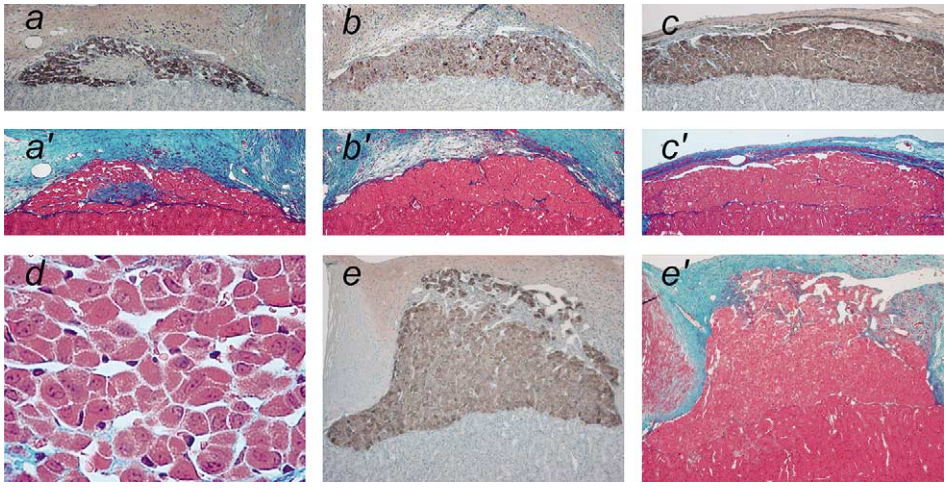

Fig. 1. Influence of adrenalectomy on the histological structure of adrenocortical cell transplants. Bovine adrenocortical cells were transplanted beneath the kidney capsule of *scid* mice. Animals were not adrenalectomized at the time of cell transplantation. At 35 days, animals were sacrificed (a and a′) or were adrenalectomized and sacrificed 24 h (b and b′) or 42 days later (c, c′, e and e′). (a–c and e) Adrenocortical cells in the transplants were distinguished from host tissue using an antibody against a mitochondrial protein (see text). (a′–c′ and e′) Tissues were stained with Masson's trichrome, which stains connective tissue blue. Note the presence of scar tissue in (a), which does not react with the anti-mitochondrial antibody. (d) High power view of part of the transplant in a non-adrenalectomized animal stained with Masson's trichrome. Magnification: (a–c and e) 40×; (d) 400×.

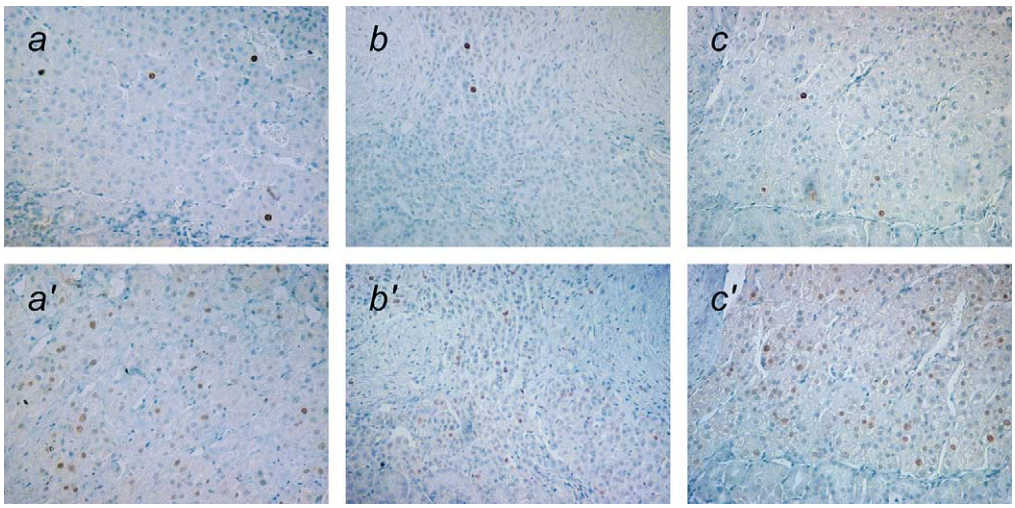

Fig. 2. Cell division and expression of $p21^{WAF1/CIP1/SDI1}$ in bovine adrenocortical cell transplants. The mouse kidney is at the bottom of each micrograph. (a and a′) Control transplant tissue; the animal was adrenalectomized at the time of cell transplantation and was sacrificed at 35 days. (b and b′) Transplant tissue in a non-adrenalectomized animal, sacrificed at 35 days. (c and c′) Transplant tissue in an animal adrenalectomized at 35 days and sacrificed 35 days later. Sections were stained with an antibody against the Ki-67 proliferation marker (a–c) or with an antibody against p21 (a′–c′). Magnification: 100×.

transplantation), in a transplant in a non-adrenalectomized animal, and in a transplant in an animal subjected to delayed adrenalectomy. Although we did not have sufficient numbers of animals in the different treatment groups to do a statistical comparison, the numbers of dividing cells appeared to be somewhat lower in the non-adrenalectomized animals. In the delayed adrenalectomy animals, the numbers were similar to those previously observed using our standard protocol [11].

In previous experiments, we also investigated the expression of $p21^{WAF1/CIP1/SDI1}$, an important inhibitory regulator of cell proliferation. Paradoxically, expression of p21 was positively correlated with cell proliferation in transplant tissues [11]. In these experiments, we also examined p21 levels by immunohistochemistry (Fig. 2). p21 was readily detectable in control transplants in adrenalectomized animals, but was substantially lower in non-adrenalectomized animals. In animals subjected to delayed adrenalectomy, the levels were much higher (Fig. 2).

In adrenalectomized animals bearing bovine adrenocortical cell transplants, we can assess the function of the transplants by measuring cortisol in the plasma [2,3]. We use cortisol levels in plasma to assess the function of the transplanted cells because the mouse adrenal gland secretes corticosterone rather than cortisol. This assay fails in the presence of the high levels of corticosterone in animals with intact adrenal glands because of the cross-reaction of corticosterone in the radioimmunoassay for cortisol. However, information on the level of function of the transplants can be obtained in animals following removal of the animals' adrenal glands. Corticosterone disappears rapidly from the plasma after removal of the animals' adrenal glands. We found that corticosterone was reduced to less than 2% of that in intact animals, 12 h after adrenalectomy. The remaining level is insufficient to affect the radioimmunoassay for cortisol.

After adrenalectomy at post-transplantation day 7 or 21, cortisol levels rose progressively (Fig. 3). Several weeks after adrenalectomy cortisol had risen to ∼100 nM. In animals that are adrenalectomized at the time of cell transplantation, plasma cortisol plateaus at ∼200 nM [3]. Additionally, we showed previously that transplants of primary bovine adrenocortical cells normally secrete aldosterone [3]. When animals were adrenalectomized 7 days after cell transplantation, aldosterone levels rose progressively to ∼100 pM. This is substantially less than the levels observed in animals that were adrenalectomized at the time of transplantation, in which levels were ∼500 pM [3]. However, when adrenalectomy was performed at 21 days following cell transplantation or later, no circulating aldosterone was detectable, even after several weeks (Fig. 3, and other data not shown).

When adrenalectomy was performed at 35 days, cortisol levels were readily detectable even at 12 h following surgery and showed a peak at 36 h; at 48 and 72 h, they were lower. By 15–30 days following adrenalectomy, levels had plateaued at ∼100 nM. Aldosterone was not detected in any animal that was adrenalectomized at 35 days.

4. Discussion

In this adrenocortical cell transplantation model system, we can distinguish various phases of the establishment and maintenance of the transplant tissue, including the initial aggregation of the cells within the host, invasion of the aggregate by endothelial cells, formation of a vascular system united with the host, and the subsequent survival, growth and function of the transplant tissue [1]. In the present

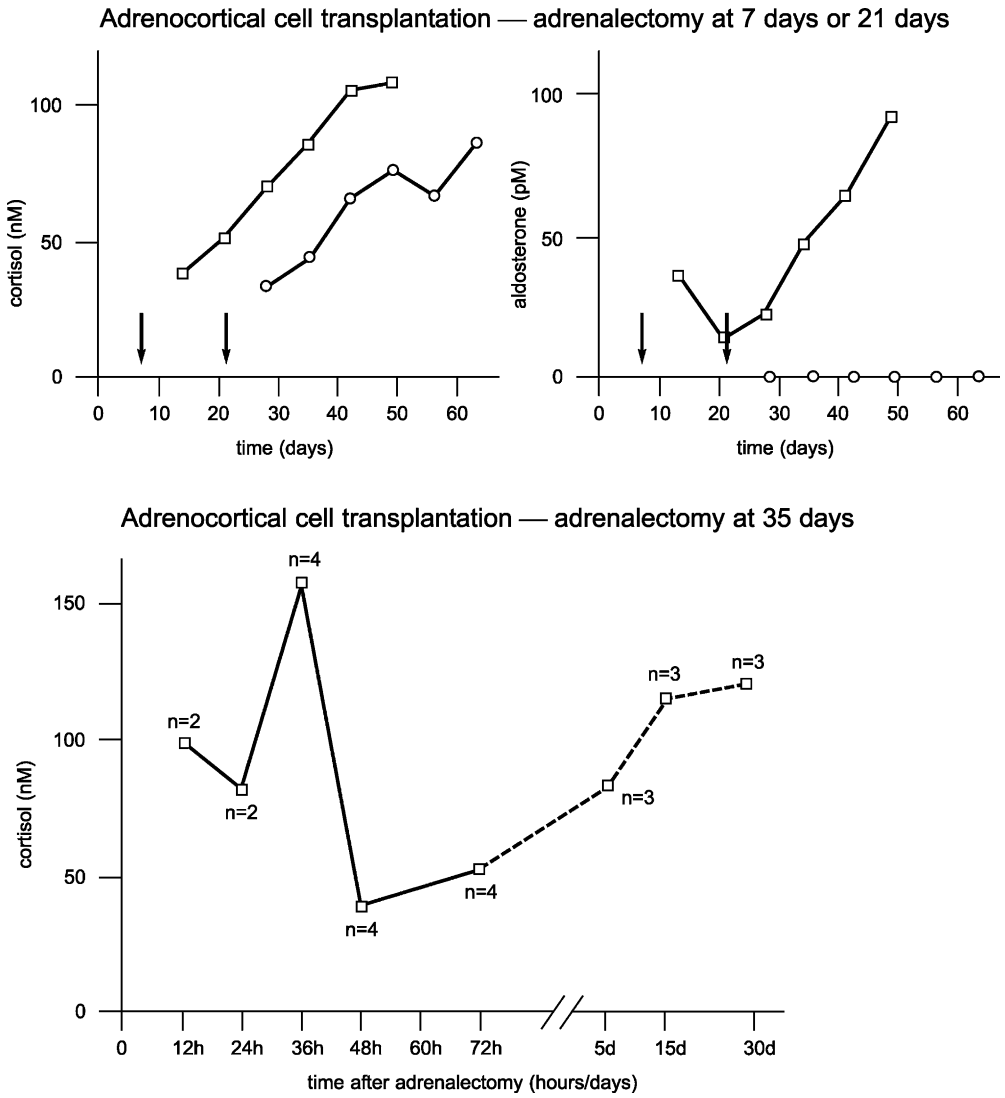

Fig. 3. Time course of changes in plasma cortisol and aldosterone in two animals that received transplanted bovine adrenocortical cells and were subjected to delayed adrenalectomy (arrows: days 7 and 21) (above). Time course of changes in plasma cortisol in a series of animals that received transplants of bovine adrenocortical cells and were adrenalectomized at day 35 (below). The animals were then sacrificed at the indicated times after adrenalectomy. The data are the averages of the plasma levels from the indicated numbers of animals sacrificed at each time point.

experiments, we investigated to what extent these processes are dependent on pituitary function, as influenced by steroid secretion by the host animals' adrenal glands. Somewhat surprisingly, in view of some data in the older literature, we found that transplants formed in non-adrenalectomized animals, although small, had a relatively normal appearance. Capillaries were easily visible in the tissue (see Fig. 1d). This indicates that the initial steps in the formation of the tissue do not appear to be dependent on elevated pituitary function. However, both the transplants themselves and the cells within the transplants were smaller than usual. This is consistent with a model in which the growth and function of the transplant is dependent on pituitary function at a stage beyond the initial phase of formation of the transplant tissue. The transplant remains capable of normal cortisol secretion following delayed removal of the animals' adrenal glands.

Despite the smaller size of the transplants in the non-adrenalectomized animals, we detected dividing cells 35 days following cell transplantation. As we noted previously, there is a remarkable decline in the proliferation rate of bovine adrenocortical cells as they are removed from cell culture and placed in the host animal [11,13]. However, the rate does not fall to zero but remains a detectable level at least to ~60 days following cell transplantation. The factors that drive this proliferation in the transplants have not yet been elucidated. The classical view is that cell proliferation in the adrenal cortex is driven by circulating ACTH [14]. ACTH levels would be expected to be much higher in animals following adrenalectomy, and we confirmed this in a small number of animals in these experiments (data not shown). However, the manner by which ACTH affects proliferation in the adrenal cortex is still uncertain, as ACTH

does not act as a direct mitogen in cultured bovine and human adrenocortical cells [14].

Expression of the cell cycle inhibitor p21 differed in transplants in non-adrenalectomized animals. We previously determined that, paradoxically, p21 expression is positively correlated with cell division in transplant tissues [11]. We speculated that p21 might be elevated by the interplay of conflicting positive and negative cell division stimuli [11]. Interestingly, p21 was lower in transplants in non-adrenalectomized animals, and was increased following delayed adrenalectomy. Taken together, the data on Ki-67 and p21 suggest that there is a lower, but non-zero, stimulus to cell division exerted on the cells in transplants in non-adrenalectomized animals, and that this stimulus is increased following delayed adrenalectomy.

The data do not allow an unambiguous determination of the level of function of the transplants in the non-adrenalectomized animals. For technical reasons, as explained in Section 3, we are unable to reliably measure cortisol, a marker for transplant function, in the presence of the high levels of corticosterone in the animals with intact adrenal glands. However, the high levels of plasma cortisol even at short periods following adrenalectomy demonstrate that, even if the transplants are not steroidogenically active in the presence of the host animals' adrenal glands, they retain the steroidogenic enzymatic machinery that enables a rapid increase in steroid output. Therefore, we can conclude that whereas cells transplanted into the non-adrenalectomized animal do not show the morphological features of high trophic stimulation (larger cell size), some level of function is nevertheless maintained by the normal (non-elevated) levels of trophic factors. Trophic factors would presumably include pituitary factors (ACTH and possibly others) and the renin/angiotensin system.

The behavior of aldosterone levels in late adrenalectomy experiments was remarkable. Although aldosterone progressively increased in an animal adrenalectomized at 7 days, no aldosterone was detected in animals adrenalectomized at 21 or 35 days, even after several weeks. The origin of aldosterone in transplants was discussed previously [3]. We favor the hypothesis that aldosterone derives from transplanted cells that have differentiated into a zona glomerulosa-type cell. If this is correct, the failure of aldosterone synthesis in delayed adrenalectomy animals suggests that these transplants lack the capacity for differentiation of cells to a zona glomerulosa type, whereas this capability exists in early adrenalectomy animals. Clearly this hypothesis requires much further study.

In summary, we can divide the process of establishment of adrenocortical tissue following cell transplantation into two phases, an initial phase in which events are dependent on the cells present in the transplant site but less dependent on circulating hormones, and a later phase in which tissue structure and function are dependent on pituitary and other endocrine influences. Future studies will address the many remaining questions regarding these two phases of adrenocortical cell transplantation.

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