Transplantation of bovine adrenocortical cells encapsulated in alginate

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Current treatment options for adrenal insufficiency are limited to corticosteroid replacement therapies. However, hormone therapy does not replicate circadian rhythms and has unpleasant side effects especially due to the failure to restore normal function of the hypothalamic–pituitary–adrenal (HPA) axis. Adrenal cell transplantation and the restoration of HPA axis function would be a feasible and useful therapeutic strategy for patients with adrenal insufficiency. We created a bioartificial adrenal with 3D cell culture conditions by encapsulation of bovine adrenocortical cells (BACs) in alginate (enBACs). We found that, compared with BACs in monolayer culture, encapsulation in alginate significantly increased the life span of BACs. Encapsulation also improved significantly both the capacity of adrenal cells for stable, long-term basal hormone release as well as the response to pituitary adrenocorticotropic hormone (ACTH) and hypothalamic luteinizing hormone-releasing hormone (LHRH) agonist, [D-Trp6]LHRH. The enBACs were transplanted into adrenalectomized, immunodeficient, and immunocompetent rats. Animals received enBACs intraperitoneally, under the kidney capsule (free cells or cells encapsulated in alginate slabs) or s.c. enclosed in oxygenating and immunoisolating βAir devices. Graft function was confirmed by the presence of cortisol in the plasma of rats. Both types of grafted encapsulated cells, explanted after 21–25 d, preserved their morphology and functional response to ACTH stimulation. In conclusion, transplantation of a bioartificial adrenal with xenogeneic cells may be a treatment option for patients with adrenocortical insufficiency and other stress-related disorders. Furthermore, this model provides a microenvironment that ensures 3D cell–cell interactions as a unique tool to investigate new insights into cell biology, differentiation, tissue organization, and homeostasis.

adrenal | alginate encapsulation | cell transplantation | LHRH

Adrenal insufficiency is the failure of adrenocortical cells to produce adequate amounts of glucocorticoids and/or mineralocorticoids. These steroid hormones play a central role in the body's homeostasis of energy, salt, and fluid; thus, adrenal insufficiency is a potentially life-threatening condition. The most relevant causes of adrenal insufficiency are autoimmune disorders (up to 80%); infectious diseases; hereditary factors; traumatic, metabolic, or neoplastic conditions; or surgical bilateral adrenalectomy, sometimes due to a compulsory therapeutic strategy in the treatment of adrenal tumors or congenital adrenal hyperplasia (CAH).

CAH due to 21-hydroxylase deficiency is the most common form of inherited adrenal insufficiency, presenting with clinical symptoms of neuroendocrine perturbations, virilization, and metabolic diseases in later life. Patients may suffer from hypotensive crises, hypoglycemia, acne, and infertility (1, 2). Current options of treatment consisting of replacement therapy with glucocorticoids, mineralocorticoids, and/or androgens can reverse the symptoms only partially, exhibit the unpleasant side effects of inappropriate glucocorticoid substitution, and leave the patients without the diurnal rhythm of glucocorticoid release. Furthermore, adrenomedullary functions, including catecholamine and neuropeptide secretion, also are disrupted (2), which correlates with cardiovascular risks, hypoglycemia, and physical disability in these patients (1, 3).

Adrenal gland transplantation could and would be a desirable therapeutic alternative for these patients if it was available and practical (4). Transplanted organs restore and maintain normal hormonal levels, adequately respond to functional demands, and regulate steroid production in response to endogenous and exogenous stimulation, including the circadian rhythm of hormone secretion. However, the application of this strategy is currently extremely limited due to the lack of human donor organs, the surgical difficulties of adrenal transplantation, and the required chronic immunosuppression.

For the correction of adrenocortical insufficiency, transplantation of whole adrenal glands might not be mandatory and the transplantation of isolated adrenal cells may be sufficient. An additional advantage of transplantation using isolated cells is the availability of various immunoisolating materials and methods for immune protection of such transplants. Application of these

Significance

Adrenal insufficiency is a life-threatening disorder that requires a complex and permanent hormone replacement strategy. All current replacement schemes suffer from numerous problems, as they fail to restore circadian variations in hormone secretion. Therefore, adrenal cell transplantation could be a preferable therapeutic alternative for patients suffering from primary adrenal dysfunction. This strategy is critically limited, however, by the lack of suitable donors of human organs and the requirement of chronic immunosuppression. Transplantation of immunoisolated xenogeneic adrenal cells could and would be a promising alternative for these patients. The most significant accomplishment of this study is the creation of long-term functional and immunoisolated artificial adrenals and their transplantation into animal models of adrenal insufficiency.

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materials not only allows avoidance of chronic immunosuppression but also allows the transplantation of xenogeneic cells (5, 6).

Sodium alginate is one of the clinically approved immunoisolating biopolymers; it has already been widely used in a variety of biomedical applications (5–10). Alginates have several unique structural and chemical parameters, appropriate not only for immune isolation but also for the creation of 3D cellular scaffolds that allow artificial organs to function long term in vitro and in vivo (7, 8, 11). The potential of xenogeneic adrenocortical cells to replace adrenal gland function has already been tested in animal models but requires acquisition of immunodeficiency (12, 13). Alginate encapsulation may protect xenogeneic adrenocortical cells from destruction by immunological processes (6). Alternatively, similar to pancreatic islets (9, 14), adrenocortical cells can be transplanted within special oxygenating and immunoisolating devices, thus reducing the risk of an immunological host versus graft response.

Creating a long-lasting, immune-isolated, and functional bioartificial adrenal was the main aim of this research. The objectives of our work included testing of primary bovine adrenocortical cells (BACs) as a potential source of cells, defining optimal conditions, and long-term monitoring. Because adrenocortical cells also express receptors for luteinizing hormone-releasing hormone (LHRH), the effect of the LHRH agonist [D-Trp6]LHRH on adrenocortical steroidogenesis in encapsulated BACs (enBACs) was tested. We characterized bioartificial adrenals in vivo and investigated their functionality and efficacy after implantation into bilaterally adrenalectomized rats.

Results

Long-Term Characterization of Functionality and Viability of Primary BACs. The function of isolated adrenocortical cells was analyzed by measuring basal and adrenocorticotropic hormone (ACTH)-stimulated (3 ng/mL) production of cortisol (Fig. 1*A*). We found that functional dynamics of BACs consisted of two phases. The first phase (days 1–6) was characterized by high levels of basal and stimulated production of cortisol, reaching a maximum on days 4–6. During this phase, the stimulation index, reflecting the functional potential of BACs, reached its maximum. Furthermore, BrdU incorporation showed a 3.5-fold increase in cell proliferation on day 6 compared with day 1. During the second phase, a decline of functional activity of BACs was observed, especially in the response to ACTH, resulting in a functional degradation with a dramatic reduction of the basal cortisol release and a total loss of the ACTH-stimulated secretion of cortisol after 11 d in culture.

Increased functional activity of BACs during the first phase was also associated with a significant elevation of the oxygen consumption rate (OCR), characterizing the metabolic state of the cells (Fig. 1*B*). On day 4, the peak of functional activity of cells, levels of OCR reached almost twice the value of 1-d-old cell cultures ($1.88 \pm 0.21 \times 10^{-15}$ vs. $1.00 \pm 0.06 \times 10^{-15}$ mol·min^{-1}·cell^{-1}, $P < 0.05$). The amount of oxygen consumed by BACs is comparable to other cell types tested in our laboratory—for example, rat β cells ($2.96 \pm 0.87 \times 10^{-15}$ mol·min^{-1}·cell^{-1}) (14). ACTH stimulation led to increased oxygen consumption compared with nonstimulated cells, which, however, did not reach statistical significance.

The second phase, characterized by a decrease of functional activity of BACs, was closely associated with a significant increase in caspase activity and apoptosis. In parallel with the observed decrease in steroidogenic activity, caspase activity progressively increased more than twofold (Fig. 1*C*). Complete loss of functionality of BACs was observed on day 14–20, which corresponds to previously published data (15). Morphologically, this process was accompanied by the increased number of dead cells and the overgrowth with fibroblast-like cells (Fig. 1*D*).

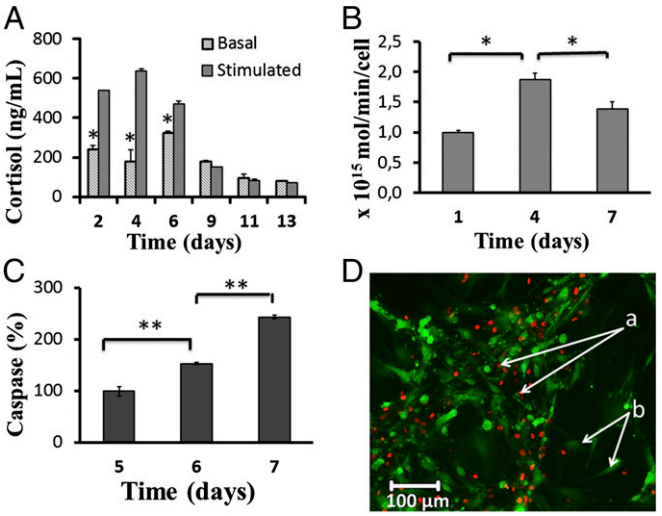

Fig. 1. Long-term characterization of functionality and viability of primary BACs. (*A*) Functional activity of free cultured cells. Basal and ACTH- (3 ng/mL) stimulated cortisol level ($n = 15$ for each value,*$P < 0.05$ basal vs. ACTH-stimulated cortisol). (*B*) OCR of free cultured cells ($n = 4$ for each value, *$P < 0.05$). (*C*) Caspase activity, reflecting intensity of apoptosis in BAC culture ($n = 3$ for each value, **$P < 0.01$). (*D*) Degradation of BAC culture on day 14 (FDA/PI staining) with increased amount of dead cells (*a*) and areas dominated by fibroblast-like cells (*b*).

Three-Dimensional Cultures of Adrenal Cells by Encapsulation in Alginate. To optimize the survival and function of adrenal cells, they were encapsulated in alginate. To identify the optimal time point for cell survival and function for BAC encapsulation, we encapsulated BACs in alginate on day 1 and on day 7 after their isolation from intact organs. The highest functional peak of cortisol (143 ± 31 ng/mL for basal and 298 ± 109 ng/mL for stimulated cortisol) was observed with the cells that were encapsulated on day 1 after isolation. With those cells, encapsulated on day 7, maximal basal cortisol reached 108 ± 18 ng/mL, and stimulated cortisol 259 ± 43 ng/mL. After a 1-month cultivation, both groups of enBACs released identical cortisol levels (basal levels of 23 ± 3.4 ng/mL for the day 1 group and 20 ± 11.6 ng/mL for the day 7 group and ACTH-stimulated levels of 103 ± 31 ng/mL for the day 1 group and 108 ± 32 ng/mL for the day 7 group). Thus, for the creation of bioartificial adrenals, cells can be encapsulated any time within the first 7 d after isolation.

In addition to the time in culture, the density of the cells encapsulated in alginate strongly affected the functionality of BACs. The optimal density was 14,000–20,000 cells per 1 µL alginate (Fig. 2*A*). Higher cell densities reduced BAC activity as a function of cell number. Alginate itself creates a diffusion barrier that plays a key role in nutrient and oxygen availability. When the thickness of the slab exceeded 500 µm (maximum 1,000 µm), cell response upon stimulation with ACTH was weaker in comparison with thinner slabs (200–500 µm). Although basal cortisol was 37 ± 12 ng/mL for both groups, stimulation with ACTH resulted in the release of 140 ± 88 ng/mL cortisol from thicker and 373 ± 62 ng/mL cortisol from thinner slabs ($n = 6$), respectively.

Interactions between different cell types within the adrenal also play an important role in the regulation of adrenocortical function (16). The influence of adrenomedullary cells on the function of adrenocortical cells is reflected in culture conditions; the steroidogenic activity of adrenocortical cells increased 10-fold by coculture with chromaffin cells (17). We assessed the impact of chromaffin cells on BAC cortisol production and viability in 3D cell culture conditions. For this purpose, chromaffin cells at a ratio of 1:5 were added to the standard number of cortical

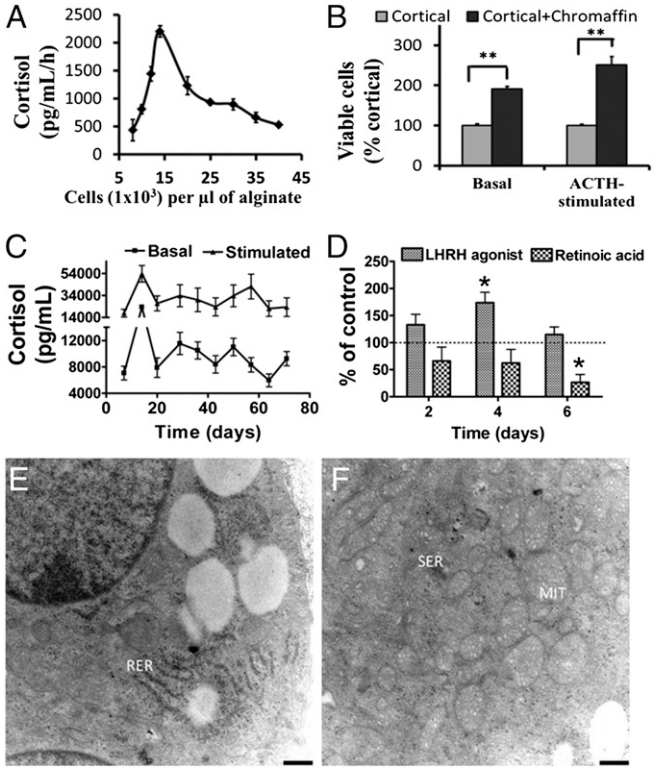

Fig. 2. Maintenance and optimization of adrenal cell survival and function by encapsulation in alginate. (*A*) Optimal density for cell survival and function in alginate ($n = 3$ for each value). (*B*) Encapsulation of chromaffin cells with cortical cells improves cell viability after 21 d of cultivation; data are shown as percentage of viable cells from pure cortical cultures ($n = 6$ for each value, $^{**}P < 0.001$). (*C*) Long-term monitoring of enBAC functionality. Cortisol levels [basal and ACTH-stimulated (3 ng/mL); $n = 18$ for each value]. (*D*) Effect of LHRH agonist [D-Trp6]LHRH and retinoic acid on basal cortisol secretion. Data are shown as percent of control ($n = 6$ for all groups, $^{*}P < 0.05$ compound vs. control). (*E* and *F*) Electron micrographs of enBACs treated with [D-Trp6]LHRH with subcellular features of stimulated cells such as ample rough endoplasmic reticulum, smooth endoplasmic reticulum, and mitochondria with a densely packed inner mitochondrial membrane.

cells. In the control group, only cortical cells were encapsulated. Encapsulation of chromaffin cells with adrenocortical cells had no influence on cortisol release, however a significant increase in BAC viability was observed (Fig. 2*B*; $P < 0.05$).

Validation of Bioartificial Adrenals: Long-Term Monitoring of Functionality of BACs Encapsulated in Alginate in Vitro. Dynamics of the functional activity of adrenocortical cells in 3D matrix over 70 d are presented in Fig. 2*C*. In the first days of culture, cortisol release from enBACs increased, with a peak on day 14 followed by a sharp decrease. In contrast to BACs in monolayer culture, functional activity of enBACs was preserved beyond 14–20 d in culture. enBACs had a long phase of stable cortisol production, which continued until the end of the observation period of 70 d. Moreover, unlike cells in free culture, enBACs did not lose their ability to respond to ACTH stimulation (Fig. 2*C*).

Another feature of enBACs was a cyclic pattern of cortisol release (Fig. 2*C*). Following the end of the first cycle at day 20, a second cycle started with a statistically significant increase and decrease in cortisol levels. Functional cycles of enBACs repeated, albeit with a slightly lower amplitude during the whole observation period (70 d). The accuracy of cycling was statistically confirmed by a Wilcoxon signed-rank test for related samples ($P < 0.05$). The frequency of cycling was ~20 d.

Impact of LHRH Agonist [D-Trp6]LHRH and Retinoic Acid on enBACs. Adrenocortical cells express receptors for both LHRH and retinoic acid (18, 19). The LHRH agonist [D-Trp6]LHRH is clinically approved for the treatment of hormone-dependent cancers, predominantly prostate cancer. Over the course of treatment, [D-Trp6]LHRH causes a surge of testosterone release at the beginning and then a reduction to levels similar to postcastration levels approximately 4 wk after injection (20, 21). Retinoic acid is involved in the regulation of steroidogenesis, and its use significantly reduces cortisol secretion (19). [D-Trp6]LHRH and retinoic acid both influenced basal (Fig. 2*D*) and ACTH-stimulated cortisol secretion from enBACs. Similar to its effect on testosterone-producing cells, [D-Trp6]LHRH significantly increased basal cortisol production by 74% on day 4 after the beginning of treatment. Stimulated adrenocortical cells are characterized by specific subcellular features reflecting steroidogenesis, such as an increased amount of inner mitochondrial membrane and endoplasmic reticulum (22). Similarly, enBACs treated with [D-Trp6]LHRH presented with ample smooth and rough endoplasmic reticulum and mitochondria with a densely packed inner mitochondrial membrane (Fig. 2 *E* and *F*). In contrast to [D-Trp6]LHRH, treating the cells with retinoic acid for 6 d significantly decreases basal cortisol secretion levels to 26% of control levels. Forty-eight hours following discontinuation of retinoic acid and [D-Trp6]LHRH application, cortisol release had returned to basal levels with no difference compared with the control group.

In Vivo Application of Bioartificial Adrenal for Glucocorticoid Replacement Therapy. In vivo testing was performed in adrenalectomized rats ($n = 15$)—4 immunodeficient and 11 normal immunocompetent animals.

Efficiency of enBACs was first assessed by transplantation into athymic, adrenalectomized Rowett Nude (RNU) rats. The control group of animals received 1×10^7 free adrenocortical cells, injected under the kidney capsule. The experimental group received the identical number of cells encapsulated in alginate enBACs, placed in the subcapsular space of the kidney. The animals underwent clinical observation for 25 d. Every second day, the functionality of the graft was determined by measuring cortisol levels in blood samples taken from the tail tip. Control animals, after an initial weight loss (6–8%) during the first 5 d after surgery, showed a stable weight gain, reaching 110–115% of the initial weight by the end of the observation period. The animals were lively; no abnormal behavior was observed. Postoperatively, blood cortisol levels increased 6–10 times on day 7 compared with the reference period 2 and 4 d postoperation (3,857 ± 1,057 pg/mL vs. 209 ± 70 pg/mL). Dynamics of cortisol concentration displayed a tendency to decrease after reaching a peak on day 11. Statistically this trend was characterized by a low coefficient of negative Spearman's rank correlation between length of transplantation and cortisol levels ($r = -0.32$, $P > 0.05$). RNU rats with implanted enBACs presented with a better postsurgical recovery and weight gain than those with transplanted free cells. They reached their initial weight 7 d after surgery, whereas rats with transplanted free cells had regained their initial weight only by the 13th day. These positive clinical differences in the postoperative status were also associated with higher blood cortisol levels. Average cortisol concentrations in animals with enBACs were 9,195 ± 2,053 pg/mL, 2.5 times higher than the levels of the control group. Remarkably, in contrast to the downward trend of cortisol levels in the group that received free cell transplantation, blood cortisol levels in animals with implanted enBACs demonstrated a progressive increase during the entire observation period ($r = +0.93$, $P < 0.05$), reaching 16 ng/mL on day 25 postimplantation (Fig. 3*A*). At the explantation of enBACs, a thin membrane of connective tissue surrounding the slabs, heavily penetrated by blood vessels, was observed (Fig. 3*B*). Explanted enBACs preserved functional, viable adrenocortical cells (Fig. 3*C*), as proven by the

release of 95 ng/mL cortisol per 1×10^7 cells after 2 h of continuous stimulation with ACTH (3 ng/mL).

To test the immunoprotective properties of encapsulation compared with immunoisolating devices, enBACs were transplanted into immunocompetent animals (Wistar rats) without the use of immunosuppressive drugs. Before starting the experiments, basal corticosterone levels of the rats were 93 ± 41 ng/mL and ACTH-stimulated levels 167 ± 49 ng/mL. After adrenalectomy, the animals were divided into three groups. The first group of animals ($n = 4$) received no treatment and was used as a control. The second group of rats received 1×10^7 enBACs i.p. ($n = 4$). The third group received 1×10^7 cells implanted s.c. in oxygenated immunoisolating devices ($n = 3$). The survival curves for the first and second group are presented in Fig. 4*A*. All animals without BAC transplantation (control) died within 12 d after adrenalectomy. These rats exhibited severe signs of adrenal insufficiency, with a progressive weight loss (Fig. 4*B*), decreased motor activity, and apathy. All animals receiving i.p. enBAC implants survived until the end of the observation period (21 d). Their body weight increased after a slight decline during the first 3 d (Fig. 4*B*). The rats were active without symptoms of apathy. These positive clinical signs were associated with high levels of basal and ACTH-stimulated blood cortisol (Fig. 4*C*) without a concomitant recurrence of relevant endogenous corticosterone levels (31–371 pg/mL). Average cortisol concentrations increased to 2,741 ± 723 pg/mL ($n = 24$, six samples from four animals). In addition, blood cortisol levels in these rats increased significantly by 2–3-fold, in response to ACTH stimulation, to 6,656 ± 1,295 pg/mL ($P < 0.05$). During explantation of enBACs on day 21, we found the slabs to be attached to the peritoneum (Fig. 4*D*); viability staining [fluorescein diacetate/propidium iodide (FDA/PI)] showed a high number of viable cells (more than 90% of the initial number). Preservation of cell function was confirmed by its ability to respond to stimulation with ACTH by releasing 76.4 ± 20 ng/mL of cortisol per 1×10^7 cells after 2 h of continuous stimulation with ACTH (3 ng/mL, $n = 8$).

Implantation of xenogeneic cells is associated with the risk of immune sensitization and graft rejection. Placing of xenogeneic material inside isolating devices provides additional protection from graft–host interaction. In this study, we used oxygenated immune-isolating devices, which have already undergone successful testing in the transplantation of islets of Langerhans with the goal to treat diabetic patients (5, 7, 10, 14, 23). Here, we performed a pilot study to determine the possibility of adapting this device for transplantation of enBACs. For this purpose, enBACs in devices were implanted s.c. in the dorsal area in three Wistar rats. The animals survived until the end of the experiment, but weight loss lasted longer and was more severe compared with the animals, transplanted i.p. with slab (Fig. 4*B*). The rats were less active and less mobile. These clinical differences were probably caused by the inconvenience related to the device size as well as the stress associated with the implementation of device oxygenation. Blood cortisol levels (1,251 ± 232 pg/mL) were slightly lower than in the animals with i.p. enBAC implants (Fig. 4*C*). To a large extent, this can be explained by the presence of the immunoisolating double polytetrafluoroethylene (PTFE) membrane, which might form a diffusion barrier to cortisol. However, the rats responded to ACTH stimulation by a significant elevation in blood cortisol (Fig. 4*C*). Explanted enBACs preserved a high viability (Fig. 4*E*) and functionality as confirmed by the production of 114 ± 28 ng/mL of cortisol per 1×10^7 cells after 2 h of continuous stimulation with ACTH (3 ng/mL, $n = 3$).

Discussion

Primary cultures of BACs have been a widely used model to study different aspects of adrenal function in vitro and in vivo (16, 24). Moreover, bovine adrenal can also be a cell source for xenogeneic transplantation in the treatment of adrenocortical deficiency. Here we show that a bioartificial adrenal gland can be created from BACs by culturing them in a 3D alginate matrix. This matrix protected transplanted cells from the host immune system in immunocompentent rats; the transplanted cells were active and rescued the animals from the lethal effects of adrenalectomy by restoring normal glucocorticoid levels.

The prerequisite to create an artificial adrenal is the capacity for long-term culture of adrenocortical cells and their ability to produce adrenocortical steroids that are secreted in a normal manner upon specific stimulation. In the present study, BACs in long-term primary monolayer culture (free BACs) underwent a process of phenotypic changes leading to loss of steroidogenic activity. As previously shown, this process is due to a loss of expression of steroid hydroxylases during prolonged cellular culture (15). Furthermore, culture of free BACs was characterized by increased apoptosis, cell degradation, and a limitation of life span.

A number of studies indicate the importance of 3D structure for cell–cell interactions, particularly for encapsulation in alginate (6, 11). Our in vitro data show that encapsulation in alginate, indeed, provides adrenocortical cells with 3D culture conditions, which prevent the loss of steroidogenic activity. The functional capacity significantly exceeded that of traditional 2D cell culture. This discovered ability of enBACs for long-term function in vitro as well as the potential for regeneration or restoration of impaired function suggests the suitability of a 3D alginate matrix for the creation of a bioartificial adrenal gland.

Such an artificial adrenal can open new medical horizons for studying the complex intraadrenal cell–cell interactions and would implement effective investigations and primary screenings of pharmacological agents. Here we show that the LHRH agonist [D-Trp6]LHRH significantly increased basal and ACTH-stimulated cortisol secretion from enBACs, indicating its potential in activating adrenocortical steroidogenesis in this artificial gland. The clinically approved drug [D-Trp6]LHRH thus might deliver the potential to improve steroidogenesis in transplanted adrenocortical

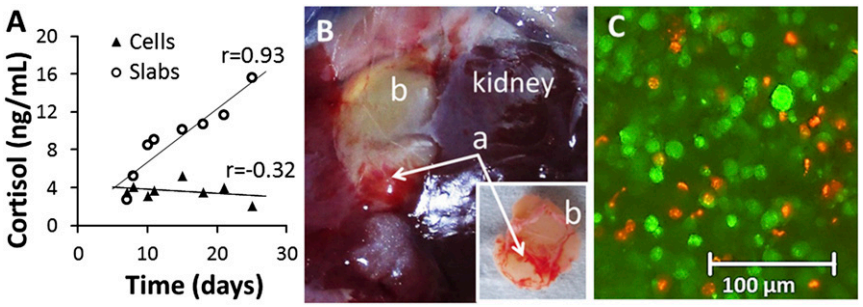

Fig. 3. Application of bioartificial adrenal for glucocorticoid replacement therapy in vivo. Trial was on RNU rats. (*A*) Linear trendline of blood cortisol. (*B*) Highly vascularized slab (*a*) before and (*b*) after explantation. (*C*) High cell viability after explantation (FDA/PI staining; green, live; red, dead).

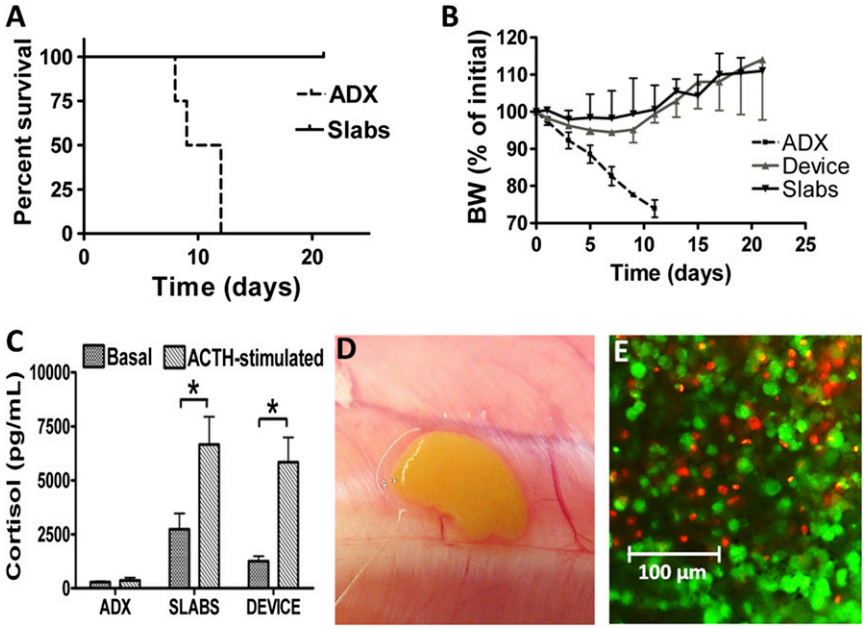

Fig. 4. Application of bioartificial adrenal for glucocorticoid replacement therapy in vivo. Trial was on Wistar rats. (*A*) Survival curve of Wistar rats. (*B*) Body weight monitoring of Wistar rats [$n = 4$ for adrenalectomized (ADX) and animals that received enBACs (Slabs) and $n = 3$ for the rats that received an implanted device]. (*C*) Blood cortisol of Wistar rats (from four ADX animals; $n = 15$ for basal cortisol and $n = 4$ for ACTH-stimulated), with slabs ($n = 24$ for basal cortisol and $n = 12$ for ACTH-stimulated—from four animals), and of animals with implanted devices ($n = 18$ for basal and $n = 9$ for stimulated blood cortisol from three rats; $*P < 0.05$). (*D*) The i.p. transplanted slab attached to peritoneum at explantation. (*E*) Live/dead (FDA/PI) staining of explanted enBACs from device (green, live cells; red, dead cells).

cells. Such an artificial adrenal might also allow the in vitro study of adrenocortical stem and progenitor cells that, so far, were impossible to isolate and culture, probably due to the inability to reconstruct the 3D in vivo environment in a culture dish (25). Most importantly, the creation of an artificial adrenocortical tissue with long-term stability opens the possibility for the transplantation of these cells with the goal of treating patients with adrenocortical insufficiency or adrenal hyperplasia due to 21-hydroxylase deficiency.

In a first set of experiments, we tested the potential of transplanted enBACs in adrenalectomized athymic, T-cell–deficient nude rats (RNU rats) to replace the host's glucocorticoid production. In accordance with our in vitro data indicating an increased functional activity and longevity of enBACs compared with free BACs, rats that received enBAC transplants presented with faster recovery, more rapid weight gain, and higher blood cortisol levels compared with adrenalectomized rats receiving free cells. In addition to the supportive function of the alginate matrix in preserving the steroidogenic function of the cells, the rich capillary network formed around the transplanted slabs might play an important role in preserving functional viability of the cells. The development of vascularization at the site of transplantation is important for the survival, growth, and function of transplanted tissue; it provides access to nutrients and is associated with stable levels of blood cortisol (26).

In a second set of experiments, we then addressed the question of whether encapsulation of BACs isolates and protects the cells from the immune system of the host. We compared the transplantation of enBACs alone into immune-competent Wistar rats with the transplantation of BACs in an immune-isolating device that has already been used successfully to transplant pancreatic islets of Langerhans without the use of immunosuppressive drugs. This device has been used both in animal models (5, 10, 14, 23) as well as in a patient suffering from diabetes type 1 (7). enBACs in all groups of adrenalectomized animals were functional, replaced corticosteroids, and rescued the animals from the lethal effect of adrenalectomy.

Transplanted enBACs were isolated from the recipient's immune system, whereas low-molecular-weight substances (nutrients, electrolytes, oxygen, and bioactive secretion products) freely diffuse through the micropores of the alginate (6). Thus, transplanted enBACs maintained their functional activity and viability throughout the observation period without the use of immunosuppression. Daily external oxygenation of the device helped to maintain cellular viability and endocrine function of the bioartificial adrenal as previously demonstrated in our diabetes models and patients (5, 7). After explantation of enBACs, a light transparent membrane with a small amount of cellular elements and a rich network of capillary vessels was found around the slabs. Absence of fibrosis around enBACs in our experiments can be understood to mean that the main secretory product of BACs is cortisol, which has well-known fibroblast-suppressive and immunosuppressive properties (27). The formation of such an abundant capillary network around the artificial adrenal might be stimulated by the recently discovered unique ability of steroid-producing cells to release a specific angiogenic factor, the endocrine-gland–derived vascular endothelial growth factor (28). The ability of enBACs to suppress fibrosis, on one hand, and to stimulate development of a capillary microenvironment, on the other hand, opens new prospects for the use of cortisol-producing cells not only in the treatment of adrenal insufficiency but also for combined transplantations, for example, together with islets of Langerhans.

Current treatment options for adrenal insufficiency or CAH are limited to glucocorticoid, mineralocorticoid, and DHEA replacement. Glucocorticoids are secreted following a circadian rhythm with the highest peak in the morning and the nadir at night. This circadian rhythm is complicated to replicate by current enteral/parenteral replacement therapies with synthetic glucocorticoids, and patients thereby suffer from a poor quality of life and increased mortality (29). Even more challenging is the treatment of adrenal insufficiency in childhood, where daily doses must be additionally adjusted to growth.

Adrenal insufficiency due to CAH is mostly diagnosed in early childhood. Patients with CAH have, in addition to the deficiency of glucocorticoids and mineralocorticoids in adolescence, an increased risk of hyperandrogenism and thus require higher doses of glucocorticoids, which often leads to loss of height and shortened adult stature (30). Thus, adrenal cell transplantation and the restoration of normalized HPA-axis function would be a particularly beneficial option for these patients. Furthermore, it may prevent adrenal crisis.

The results of the present study indicate that BACs encapsulated in alginate are suitable for replacement therapy of adrenocortical insufficiency not only by their direct i.p. administration to a recipient but also when implanted in a special oxygenated immunoisolating device. Direct transplantation of immune-isolated xenogeneic cells in biomatrices undoubtedly has great prospects. However, the widespread clinical application of this transplantation method is currently limited by inevitable risks from a direct contact of the host's immune system with the xenogeneic material. Biomatrix degradation may potentially lead to the escape of breakdown products of the matrix and the development of sensitization to the newly immunologically exposed cells. In this regard, along with the creation of artificial endocrine organs on the basis of encapsulated primary xenogeneic cells in 3D scaffolds, their additional protection within oxygenated immunoisolating devices is a further promising approach to the regenerative therapy of patients with adrenal insufficiency or other polyglandular autoimmune insufficiencies.

Materials and Methods

The complete description of the methods is in *SI Text*.

Cell Preparation and Culture. BACs and chromaffin cells were isolated from bovine adrenals of freshly slaughtered 1–3-y-old cattle by collagenase digestion, as previously described (17, 31, 32).

Cell Encapsulation. Pelleted BACs were gently mixed with 3.5% (wt/vol) sterile high guluronic acid (HG) alginate, dissolved in Custodiol-HTK solution (H.S. Pharma). The alginate–cell mixture was then placed either on a glass (for slabs) or spread in the cell compartment of the chamber device (10). Then alginate was cross-linked by applying flat Sintered glass (Pyrex), saturated with 70 mM strontium chloride plus 20 mM Hepes. The thickness of alginate/cell slab was 550 μm.

Experiments with the LHRH Agonist [D-Trp6]LHRH and Retinoic Acid. The LHRH agonist [D-Trp6]LHRH, dissolved in DMSO, was used at 10^{-6} M/L; retinoic acid, dissolved in ethanol, was applied at a concentration of 5 μM/L.

In Vivo Sudies. Female RNU (8 wk old) and female Wistar rats (200 g) were obtained from Charles River Laboratory.

Adrenalectomy and Implantation. Bilateral adrenalectomy was performed simultaneously with the cell transplantation procedure. For naked cell transplantation, 5×10^6 of bovine adrenocortical cells were infused into a pouch formed under the capsule of each kidney. For encapsulated cell transplantation, two identical slabs were implanted, one underneath each kidney capsule. For i.p. transplantation in adrenalectomized Wistar rats, two slabs, containing 5×10^6 cells each, were carefully placed into the retroperitoneal space. Macrochambers were placed under the dorsal skin. An oxygen-enriched gas mixture (60% oxygen, 35% nitrogen, 5% CO_2) was used for daily "refueling" of the gas module.

Ethics Statement. All animal experiments were performed in strict accordance with animal protocols approved by the ethical and research board of the Regierungspräsidium Dresden.

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