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Transplantation of Adrenal Cortical Progenitor Cells Enriched by Nile Red

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Abstract

Background—The adrenal cortex may contain progenitor cells useful for tissue regeneration. Currently there are no established methods to isolate these cells.

Material and Methods—Murine adrenal cells were sorted into a Nile-Red-bright (NR^{bright}) and a Nile-Red-dim (NR^{dim}) population of cells according to their degree of cholesterol content revealed by Nile Red fluorescence. The cells were transplanted under the renal capsule to determine their ability for regeneration.

Results—The NR^{bright} cells contained an abundance of lipid droplets, whereas the NR^{dim} cells contained little. The NR^{bright} cells expressed *Sf1* and the more differentiated adrenal cortical genes including *Cyp11a1*, *Cyp11b1*, and *Cyp11b2*, whereas the NR^{dim} cells expressed *Sf1* but not the more differentiated adrenal cortical genes. After 56 days of implantation in unilateral adrenalectomized mice, the NR^{dim} cells expressed *Sf1* and the more differentiated adrenal cortical genes, whereas the NR^{bright} cells ceased to express *Sf1* as well as the more differentiated adrenal cortical genes. NR^{dim} cells also proliferated in the presence of basic fibroblast growth factor.

Conclusions—The population of NR^{dim} cells contained adrenal cortical progenitor cells that can proliferate and give rise to differentiated daughter cells. These cells may be useful for adrenal cortical regeneration.

Keywords

Nile Red; adrenal cortex; regeneration; progenitor cells

INTRODUCTION

The existence of stem cells in the adrenal cortex has been suggested by several previous studies (1-8). In rats, a zone of cells located between the zona glomerulosa and the zona fasciculata does not express either aldosterone synthase or 11- β 1-hydroxylase, enzymes specific for the glomerulosa and fasciculata, respectively(9). Cells in this zone actively synthesize DNA as demonstrated by the uptake bromodeoxyuridine into the nuclei (9,10). Such labeled cells migrate toward the adrenal capsule and medulla over a period of several days. Additional

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evidence for adrenal cortical stem cells comes from enucleation experiments where the bulk of the adrenal cortex is removed (11). Cells in the remaining adrenal capsule can regenerate the adrenal cortex in a centripetal pattern (12). Cells isolated from either the bovine or the human adrenal cortex can also reconstitute the adrenal cortical function (13-16); however, the stem cell responsible for the adrenal cortical regeneration has not been isolated or characterized.

During development, the steroidogenic factor 1 (*Sf1*) becomes expressed in cells that are destined to become the adrenal cortex (17-19). Postnatally, cells in the functional zones of the adrenal cortex synthesize steroid hormones from cholesterol. A series of genes encoded by *StAR*, *Cyp11a1*, *Cyp11b1*, and *Cyp11b2* become differentially expressed in the functional zones. Most of these genes encode enzymes that are needed for steroidogenesis. For example, *Cyp11b1* encodes 11- β 1-hydroxylase, which is present in the zona fasciculata, and *Cyp11b2* encodes aldosterone synthase, which is present in the zona glomerulosa.

If the adrenal cortical stem cells do not express the necessary enzymes for steroidogenesis, then they should contain less cholesterol compared to the more differentiated adrenal cortical cells from the functional zones. While adrenal cortical stem cells can self-renew indefinitely, adrenal cortical progenitor cells have limited proliferative potential and give rise to the differentiated cells in the zona glomerulosa and the zona fasciculata. We hypothesize that the adrenal cortical progenitor cells may be enriched by examining the intracellular storage of cholesterol, which becomes esterified and is stored as cytoplasmic lipid droplets (20). These cytoplasmic lipid droplets can be localized and quantified by their interaction with Nile Red (21), a hydrophobic phenoxazone dye that intensely fluoresces in the presence of lipids (22, 23). Nile Red has been utilized as a sensitive fluorescent histochemical stain for tissue lipids and for fluorescent activated cell sorting (FACS) (22-25). In this study, we demonstrate that cells in the adrenal gland can be separated into two groups of cells based on their affinity for Nile Red. Furthermore, the group of cells with the weak Nile Red affinity contains the adrenal cortical progenitor cells.

MATERIALS AND METHODS

Reagents and media

Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12), Hank's balanced salt solution (HBSS), fetal bovine serum, horse serum, and antibiotics were purchased from Invitrogen (Carlsbad, CA). Collagenase I, deoxyribonuclease I, and bovine serum albumin were purchased from Sigma-Aldrich Corp (St. Louis, MO). Nile Red was purchased from Molecular Probe (Carlsbad, CA). Collagen solution, PureCol, was purchased from Inamed Biomaterials (Fremont, CA). Collagen sponge Helistat was purchased from Integra (Plainsboro, NY).

Animals

Female C57/BL6 mice of eight weeks old (20~22 g) were purchased from Jackson Laboratory (Bar Harbor, MA). All animals were maintained in an animal barrier as a non-breeding colony in a temperature and light controlled room and allowed free access to food and water. In each experiment, mice from the same strain acted as both donor and recipient of transplants. The use of the animals was approved by the UCLA Animal Research Committee. Isolation of adrenal cells

For each preparation, 10 murine adrenal glands were removed from animals after euthanasia. After removing the surrounding fat, they were incubated in the digestion mixture at 37°C for 1 hour with gentle shaking. The digestion mixture consisted of 10 ml of HBSS containing 2 mg/mL collagenase I, 0.05 mg/mL DNase I, and 5 mg/mL bovine serum albumin. After

dispersing the cells through a pipette, they were washed and filtered through a 40- μ m strainer (Millipore, Bedford, MA) and were counted with a hemacytometer. The viability was assessed by Trypan Blue exclusion. Cells were cultured in the serum-free Knock-Out (KO) medium supplemented with 100 ng/mL of basic fibroblast growth factor (bFGF) in tissue-culture-treated polystyrene plates (Corning, Corning, NY).(26). The numbers of cells in each well were quantified by the CyQUANT Cell Proliferation Assay Kit purchased from Invitrogen (Carlsbad, CA) following the manufacturer's protocol. For cells cultured in vitro, CyQUANT[®] GR was directly mixed into each well before transferred into a 96-well microplate. The mixture was then placed into a microplate reader to be read at 480 nm excitation and 520 nm emission along with a standard curve produced by serially diluted concentrations of bacteriophage λ DNA (Invitrogen, Carlsbad, CA) and adrenal cortical cells. The resulting fluorescence values were converted into cell numbers by referencing the standard curve. Attachment was calculated by dividing the number of cells attached after 24 hours by the number of cells originally seeded into each well. Cell growth was expressed as fold increase and was calculated by dividing the number of cells on day 7 by the number of cells attached after 24 hours.

Nile Red staining

Nile Red solution (1 mg/ml in DMSO) was prepared and stored protected from light in a -20°C freezer. Staining was carried out on both fixed tissue and cells, and on unfixed cells. Optimum cutting temperature (O.C.T.) embedded adrenal glands were cut as 7- μ m sections. Cells were fixed onto glass slides by a slide warmer. The preparation was incubated for 10 minutes at room temperature. Nile Red was prepared 1:100 in 75% glycerol and was added onto the section. Cells isolated from the adrenal glands were resuspended in HBSS, and the dye was added directly to the cell suspension at 1:100 dilution. The cell suspension was incubated in a 37°C water bath for 10 minutes and was placed on ice for 10 minutes before cell sorting.

Flow cytometry

Samples stained with Nile Red were sorted on a digital FACSVantage SE flow cytometer (BD Biosciences). The argon-ion laser was tuned to 488 nm and run at 250 mW of power. Green Nile Red fluorescence was collected with a 530/30 nm band pass filter. Fluorescence data were displayed on a four-decade log scale using FACSDiVa software (BD Biosciences). Samples were discriminated on forward scatter (FSC) vs. side scatter (SSC) plots and were sorted into subpopulations based on the intensity of Nile Red staining. During sorting cellular aggregates were excluded on a SSC-area vs. SSC-width dot plot. Reanalysis of the sorted samples was performed on a BD LSRI cytometer (BD Biosciences).

Fluorescence microscopy

The fluorescence microscopy studies were carried out with a Zeiss Axioskop 2 upright microscope (Thornwood, NY) using a 120W metal halide light source by EXFO (Ontario, Canada). Nile Red fluorescence was viewed at green fluorescence, using a 515 nm long pass filter, and DAPI fluorescence was viewed at blue fluorescence using a 397 nm long pass filter. Pictures were photographed with a SPOT camera and the SPOT software purchased from Diagnostic (Sterling Heights, MI).

Implantation

After sorting, cells were resuspended and seeded onto Helistat (27). Approximately one million non-sorted, seven hundred thousand NR^{bright}, or three hundred thousand NR^{dim} cells were resuspended in 30 μ L of media containing 0.24 mg/mL of neutralized collagen before being seeded onto each collagen sponge that was 3 mm $\times$ 2 mm $\times$ 7 mm in size. Neutralized collagen was made by mixing 80 μ L of collagen with 10 μ L of 10x PBS and 10 μ L of 0.1 N sodium

hydroxide solution. The cells-collagen constructs were then placed in the incubator to allow the cells to attach for an hour before implantation. Induction of general anesthesia was performed through inhaling combination of 5% isoflurane and 1 L/min oxygen for 2 min. The dorsal skin was prepared neck through tail with three alternative scrubs of providine iodine and alcohol. Mice were positioned prone under maintenance of general anesthesia through inhaling combination of 2.5% isoflurane and 0.5 L/min oxygen. A 3-cm incision was made through dorsal midline. The subcutaneous tissue was detached bluntly. Myotomy was performed parallel to the lower edge of the liver. The left adrenal gland was identified within the space defined by the hepatic lower edge, the renal upper pole, and the spinal column. The adrenal pedicle was clamped tightly for 5 sec before adrenalectomy. The kidney was exteriorized without tension and temporarily fixed with prehydrated sterilized gauze. The ventral renal capsule was lifted away from the renal cortex through a transverse incision. The implant was inserted under the renal capsule. The kidney was replaced afterwards without torsion of the renal pedicle. Muscular layers were closed with 6-0 Prolene sutures (Ethicon, Piscataway, NJ), and the skin was sutured with 3-0 Vicryl (Ethicon, Piscataway, NJ). Mice were euthanized through inhalational isoflurane as scheduled.

RNA extraction and qRT-PCR

RNA extraction and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was done as previously described (26). Briefly, Total RNA isolation was performed following Qiagen RNeasy Mini Kit protocols and expressions of specific mRNAs were analyzed by qRT-PCR. *Sfl* was amplified by primers 5'-TGCTGGTGTGGACCACATCTA-3', 5'-CAGTAACCAGCAGGATGCTGTCT-3', and probe 5'-[6-FAM]CGCCAGGTCCAGTACGGCAAGG [TAMRA-6-FAM]-3'. *Cyp11b1* was amplified by 5'-AGAGCTGGCAGAGGGTCGT-3', and 5'-TGGCATCCATTGACAGAGTTCT-3', and probe 5'-[6-FAM]CACAGTCCTGGAGTGTACAGCAGAGCT [TAMRA-6-FAM]-3'. *Cyp11b2* was amplified by was amplified by 5'-CAGACTCGGCAGCTCTCAGA-3', and 5'-ATGGCGTCGAGAGGCAAA-3', and probe 5'-[6-FAM]CTACAGTGGCATTGTGGCGGAATAATATCTCA [TAMRA-6-FAM]-3'. *Cyp11a1* was amplified by 5'-CAACAAGCTGCCCTTCAAGAAC-3', and 5'-ACCGTGCCACCCCTCCT-3', and probe 5'-[6-FAM]CCAGGCCAACATTACCGAGATGCTGT [TAMRA-6-FAM]-3'. Rodent *GAPDH* primers/probe was purchased from Applied Biosystems (Foster City, CA). The relative level of each adrenal-specific gene was normalized to that of pooled neonatal adrenal glands for the sorted cells or to that of all adrenal cells for implantation specimens.

Statistics

Results are presented as mean $\pm$ standard deviation. Gene expressions at each time point were evaluated using ANOVA (General Linear Models, SAS/STAT, SAS Institute, Cary, NC). For measurements in which there was a significant difference, the post-hoc testing of pairwise differences between cell groups at each time point was performed using the Tukey's Studentized Range (HSD) Test (SAS/STAT, SAS Institute, Cary, NC).

RESULTS

Nile Red was used to stain cryosections of the adrenal gland to determine the tissue distribution of lipid droplets (Figure 1A). Within the adrenal cortex, the zona fasciculata showed the strongest Nile Red fluorescence, followed by the zona glomerulosa. Simultaneous nuclear fluorescence demonstrated an even distribution of cells within the adrenal gland (Figure 1B), as shown by digitization of the DAPI fluorescence (Figure 1D). The adrenal capsular cells and the medullary cells had the least amount of Nile Red fluorescence, and some fluorescence was noted in between the adrenal medulla and the adrenal cortex (Figure 1E).

Based on their differential Nile Red fluorescence, cells isolated from the adrenal gland were separated by fluorescent activated cell sorting (FACS, Figure 2A). The population of cells with the stronger intensity was labeled as NR^{bright}, and the population of cells with the intermediate intensity was labeled as NR^{dim} (Figure 2B). The NR^{bright} population consisted of 64.6±5.1% of the parent population, and the NR^{dim} population consisted of 26.5±3.7% of the parent population. When the NR^{bright} population was analyzed for its forward and side scattering properties, these cells tend to have higher side scattering (Figure 2C). In contrast, the NR^{dim} population had lower side scattering characteristics but a wider range of forward scattering (Figure 2D).

The sorted adrenal cells were observed under the microscope to confirm their Nile Red fluorescence. Both the NR^{dim} population and the NR^{bright} population were visible under the fluorescence microscope with DAPI staining. Cells in the NR^{bright} population contained bright particles of lipid droplets (Figure 3), whereas the NR^{dim} populations had few lipid droplets and was barely visible under the green fluorescence filter.

The gene expression of the sorted cells was analyzed by qRT-PCR (Figure 4). Cells isolated from adrenal glands expressed many adrenal cortical specific genes including *Sf1*, *Cyp11b1*, *Cyp11b2*, and *Cyp11a1*. Both the NR^{dim} and NR^{bright} populations of cells expressed similar levels of *Sf1* as all of the cells prior to sorting, indicating both populations contained adrenal cortical cells. In contrast, the NR^{dim} population expressed significantly lower levels of *Cyp11b1*, *Cyp11b2*, and *Cyp11a1* as compared to the NR^{bright} population, indicating the absence of adrenal cortical zone-specific markers in the NR^{dim} population.

All of the cells isolated from the adrenal gland, the NR^{bright} population of cells, and the NR^{dim} populations of cells were separately seeded onto collagen sponges for implantation studies. The implants, which appeared pale white to yellow, were retrieved after 10, 28, and 56 days. On days 10 and 28, implants seeded with the NR^{bright} cells had a similar gene expression profile as those seeded with all of the isolated cells (Figure 5). Implants seeded with the NR^{dim} cells expressed similar level *Sf1* as those seeded with all of the isolated cells but lacked significant expression of the other adrenal cortical zone-specific genes. On day 56, implants seeded with the NR^{bright} cells expressed very low levels of *Sf1*, *Cyp11b1*, *Cyp11b2*, and *Cyp11a1* (Figure 5). In contrast, implants seeded with the NR^{dim} cells had a gene expression profile comparable to those seeded with all of the isolated cells.

After one day of cell culture in KO medium, approximately 20 % of the NR^{dim} cells remained attached, whereas only 5% of the NR^{bright} cells remained. Neither cell population proliferated in the KO medium alone. In the presence of 100 ng/mL bFGF, the number of NR^{dim} cells increased 4-fold and 10-fold on day 7 and day 14, respectively (Figure 6). In the presence of bFGF, NR^{bright} cells also increased by 2-fold and 4-fold on day 7 and day 14, respectively. For both NR^{dim} and NR^{bright} cells, bFGF did not significantly affect the expression of *Sf1*, *Cyp11b1*, and *Cyp11b2* after 14 days of culture (Figure 6).

DISCUSSION

The adrenal cortical progenitor cells are located either just beneath the capsule or in between the zona glomerulosa and the zona fasciculata. Because there are no known surface markers that define these cells, the evidence for the progenitor cells' existence is largely indirect (9, 12,28,29). It is believed that the adrenal cortical progenitor cells express *Sf1* but not the zonal specific genes such as *Cyp11b1* and *Cyp11b2* that encode 11- β -hydroxylase and aldosterone synthase, respectively (9,12,29,30). Since cholesterol is the precursor for steroid hormones, the steroidogenic cells in the functional layers contain an abundance of cholesterol. In contrast, the adrenal cortical progenitor cells do not express the steroidogenic enzymes and should

therefore contain less cholesterol. If this hypothesis is correct, then the population of adrenal cells that contains less cholesterol should be enriched with adrenal cortical progenitor cells.

Both NR^{bright} and NR^{dim} cells are heterogeneous populations that may contain various cell types. In this study, we found that the NR^{dim} cells with less cholesterol did not express the zonal specific genes but did express *Sfl*. Furthermore, this population of cells was able to regenerate the zonal specific gene expression and retained *Sfl* expression after 56 days of implantation. On the other hand, the NR^{bright} cells expressed *Sfl* and the other zonal specific genes, indicating their origin from the classical adrenal cortical zones. These cells were able to function for 28 days after implantation but eventually ceased the adrenal specific gene expression including *Sfl* expression, suggesting that the NR^{bright} cells were terminally differentiated and had a limited life span after implantation. These data suggest that the adrenal cortical progenitor cells are enriched in the NR^{dim} population of cells. Although the demonstration of steroid hormone secretion from the transplanted NR^{dim} cells would be ideal, the presence of an intact adrenal gland on the contralateral side in the recipient mouse limits the utility of direct hormone measurements.

In addition to their differences in adrenal cortical gene expression, NR^{bright} and NR^{dim} cells were also different in their light scattering characteristics. Two populations of cells with distinct forward and side scatter properties were observed. In general, cells of different complexity will have different side scattering. NR^{bright} cells have high side scattering characteristics, which correlate with the abundance of cytoplasmic lipid droplets. NR^{dim} cells, on the other hand, have low side scattering characteristics.

In vitro data suggested that NR^{dim} cells attached better compared to NR^{bright} cells when cultured in the KO medium. Neither subpopulation of cells proliferated significantly without the stimulation by bFGF, which may directly or indirectly act on the progenitor cells. After being cultured in the KO medium, both subpopulation of cells expressed similar levels of *Sfl*, *Cyp11b1*, and *Cyp11b2* genes regardless of the addition of bFGF.

Stem cells are able to self-renew and give rise to differentiated daughter cells. This study did not document the presence of adrenal cortical stem cells because the unlimited potential for self-renewal was not shown. The Hornsby's group had studied adrenal cortical regeneration with several selected clones derived from human and bovine sources (16,31). These clones showed different abilities to form functional adrenal cortical tissue. However, the phenotype of the regenerating cells were not directly determined. In this study, we demonstrated that the NR^{dim} cells expressed *Sfl* but lacked the zonal specific adrenal cortical gene expression. This population of cells was able to give rise to cells that expressed the zonal specific adrenal cortical genes after 56 days of implantation. This finding implies that there is a populations of cells that can further differentiate into zonal specific adrenal cortical cells. Because the self-renewal potential of the NR^{dim} cells has not been determined, we do not know whether this population of cells contain the adrenal cortical stem cells. The NR^{dim} cells, however, do have a limited proliferative potential and can give rise to more differentiated zonal cells. Therefore, the adrenal cortical progenitor cells are enriched in the NR^{dim} cells.

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Figure 1.

Nile Red and DAPI staining of adrenal gland, 200x. 7- μ m adrenal gland sections were stained with Nile Red and DAPI to locate lipid droplets and nuclei. A. Nile Red staining of the adrenal gland showed the highest fluorescence within the zona fasciculata. B. DAPI staining of the adrenal gland showed a uniform distribution of nuclei. C. Merged picture of A and B. D and E, the fluorescent intensity of Nile Red and DAPI versus the radius of adrenal gland. m, medulla; f, zona fasciculata; g, zona glomerulosa; c, adrenal capsule.

Figure 2.

The forward scattering, side scattering, and fluorescence characteristics of Nile Red stained adrenal cells. A. Forward and side scattering of all of the isolated adrenal cells. Staining of Nile Red does not change the forward and side scattering of the cells. B. Nile Red stained adrenal cells were discriminated by the green fluorescent (530/30) channel and the dark red channel (660/20). Two distinct populations, NR^{bright} and NR^{dim}, can be observed. C. Forward and side scattering of NR^{bright} cells. D. Forward and side scattering of NR^{dim} cells.

Figure 3.

Nile Red staining of sorted adrenal cells, 200x. A-D; after sorting, sorted cells were fixed to slides and observed by a fluorescent microscope. A, D, and G, All cells. B, E, and H, NR^{bright} cells. C, F, and I, NR^{dim} cells. A, B, and C were stained with Nile Red. Lipid droplets can be observed. D, E, and F, DAPI staining of the NR^{bright} cells to locate nuclei. G, H, and I, merged picture of Nile Red and Dapi staining.

Figure 4.

Relative gene expression levels of *Sf1*, *Cyp11b1*, *Cyp11b2*, and *Cyp11a1* of all adrenal cells, NR^{bright} cells, and NR^{dim} cells. The relative level of each adrenal-specific gene was normalized to that of pooled neonatal adrenal glands. Error bar represents standard deviation of measurements of retrieved implants from three animals. The levels of *Sf1* were the same ($p>0.05$), where as the levels of *Cyp11b1*, *Cyp11b2*, and *Cyp11a1* were significantly lower for the NR^{dim} cells.

Figure 5.

Relative gene expression level of *Sf1*, *Cyp11b1*, *Cyp11b2*, and *Cyp11a1* of NR^{bright} cells, and NR^{dim} cells that were implanted into the animal after 10, 28, and 56 days. The gene expression level of NR^{bright} cells, and NR^{dim} cells was normalized to that of all adrenal cells on the same day. A. Genes expressions levels of retrieved implants at day 0. B. Genes expressions levels of retrieved implants at day 10. C. Genes expressions levels of retrieved implants at day 28. D. Genes expressions levels of retrieved implants at day 56. The relative level of each adrenal-specific gene was normalized to that of all adrenal cells for implantation specimens. * indicates $p < 0.05$ as compared to all adrenal cells, and # indicates $p < 0.05$ as compared to NR^{bright} cells. Error bar represents standard deviation of measurements of retrieved implants from three animals.

Figure 6.

The attachment of NR^{bright} and NR^{dim} cells in KO medium (A) and growth with the aid of bFGF on day 7 (B) and day 14 (C) were investigated. D, Gene expression of NR^{bright} and NR^{dim} cells immediately after cell sorting. Both types of cells expressed similar levels of *Sf1*, but NR^{dim} cells expressed significantly lower levels of *Cyp11b1* and *Cyp11b2*. Gene expression of NR^{bright} and NR^{dim} cells after 7 days (E) and 14 days (F) of culture in the KO medium. Error bar represents standard deviation of repeated measurements.