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Peter Hously 9/26/96  
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### Introduction

The aim of these studies is to provide basic information on the regulation of androgen precursor synthesis by the human adrenal cortex and to test the effects of adrenal androgens (dehydroepiandrosterone, DHEA, and dehydroepiandrosterone sulfate, DHEAS) on human breast cancer growth in a mouse model. The immunodeficient *scid* mouse is being used both as host for functional human adrenal organoids (i.e., implanted tissue structures) as a source of androgens and will have human breast cancer cells implanted as a target tissue.

Uses of the information to be obtained on human adrenocortical DHEAS production will be the identification of hormonal and molecular factors that set the adrenal androgen production level. This information may more precisely define the risk factors of adolescent and postadolescent women for higher peak levels of DHEAS and consequent increased exposure of the breast tissue to estrogens. The better characterization of the factors that regulate adrenal androgen synthesis, currently very poorly defined both molecularly and physiologically, would enable appropriate diagnosis and interventions in high-risk women and may provide other avenues of rational treatment in estrogen-responsive breast cancer.

Over the past year, the focus of this project has been on improving the in vivo model system for the analysis of the regulation of human adrenal androgen biosynthesis in the *scid* mouse. Additionally, new information on the proteins binding to the  $3\beta$ -hydroxysteroid dehydrogenase genes has been obtained.

### Body

#### *Task 1*

Further develop the human adrenal organoid/*scid* (severe combined immunodeficiency) mouse model for investigation of the regulation and effects of human adrenal androgens.

As reported in last year's Progress Report, we found that the production of the adrenal androgens, DHEA (dehydroepiandrosterone) and DHEAS

(dehydroepiandrosterone sulfate) by the adult human adrenal gland is exclusively the function of the innermost zone of the adrenal cortex, the zona reticularis (Endoh et al., 1996). The zona fasciculata does not produce DHEA or DHEAS, and produces only cortisol. Consequently, the essential feature of a model in which normal human adrenal androgen production is maintained in a mouse via an implanted human adrenal organoid is the maintenance of zona reticularis function. For this purpose, we have been developing the following approaches.

The first is to separately implant zona fasciculata and zona reticularis cells, to examine whether the separated cells maintain their individual properties when implanted back into the in vivo environment. Our data indicate, so far, that zona fasciculata and zona reticularis cells, although maintaining separate properties in short-term cell culture, both form organoids that support approximately equal plasma cortisol levels in the SCID mouse. Moreover, DHEAS levels in the plasma of the mice with human adrenal organoids were extremely low, both for organoids formed from reticularis cells and fasciculata cells. However, experiments carried out during this year have indicated that this may not necessarily reflect an inability of the organoids to produce adrenal androgens. To test this, we administered DHEA in a dimethyl sulfoxide solution intramuscularly to control mice (without cell implants) and measured the resultant DHEAS levels in the plasma over several days. It was found that administration of high doses of DHEA to the mice did not substantially increase plasma DHEAS levels. This indicates that a very high rate of DHEA or DHEAS production by an organoid may be required to produce and maintain a circulating level of DHEAS similar to the normal circulating levels of DHEAS in adult humans. There is a  $\sim 10^5$ -fold difference in plasma DHEAS levels between the adult human and the normal mouse. It is not clear whether this difference results solely from the difference in the production rates of DHEA by the adrenal cortex of the two species (the mouse synthesizes essentially none). There may be other differences such as the metabolic clearance of DHEA by the liver or excretion of DHEA(S) by the kidneys. Consequently, an organoid may have to produce very high quantities of DHEA to maintain human-type plasma levels in the mouse. Experiments previously conducted in rats (although these have not been done in mice) indicated that feeding of high levels of DHEA in food was capable of raising plasma DHEAS levels, but that a threshold level of DHEA must be

administered before plasma DHEAS was affected (Abadie et al., 1993). Thus, we conclude that the level of production of DHEA or DHEAS by an organoid must exceed some threshold level to exceed the clearance rate in the mouse. If, as is possible, both zona fasciculata and zona reticularis cells when implanted re-zone into a predominantly fasciculata cell type, then either (i) an organoid must be engineered in a manner sufficient to permit zonation with formation of a large zona reticularis; or (ii) an organoid must be formed from cells genetically engineered to produce high levels of DHEA. The latter approach is discussed further under Task 3.

Approach (i), to form organoids with adequate re-zonation into fasciculata and reticularis zones, is being investigated using several methods for cell implantation. As outlined in the previous Progress Report, we have tested three different modes of implantation of the cells. So far, the results indicate that, using primary human adrenocortical cells, survival and revascularization of the cells occurs in all of the models tested, but that the results are essentially the same in all cases; i.e. cortisol is secreted but we do not observe high plasma levels of DHEAS. We conclude that it is necessary to engineer a device in which the cells may be implanted which will permit the formation of a longer vascular bed and thereby support proper zonation; work along these lines is in progress.

#### *Task 2*

Assess the influence of circulating adrenal androgens on human estrogen response in human breast cancer cell growth.

The aim of this task is to assess the influence of adrenal androgens produced by implanted human adrenal organoids in the SCID mouse on the growth of co-implanted tumor cell cells (MCF-7). These cells have been transfected with human aromatase, to mimic the typical metabolic pathways existing in primary human breast cancer. During the past year, we have investigated the growth of aromatase-transfected MCF-7 cells in SCID mice. We have found that the cells do not grow well in the particular strain of SCID mice (ICR SCID) of which our colony is composed. We are developing two approaches to this problem. One is to attempt to use the MCF-7/aromatase cells as a transplantable tumor rather than implanting a primary cell suspension from cells grown in culture. MCF-7 has been used previously as a transplantable tumor in immunodeficient mice (Oka et al., 1996) and although this has not previously been done



with aromatase-transfected MCF-7 cells, it should in principle be feasible. This method would allow a larger, vascularized, tumor inoculum to be used, and should improve the "take" of the tumor versus the use of a nonvascularized free-cell suspension. An alternative would be to test and use a different transplantable human breast cancer line with the same desirable properties, or to use primary human breast cancer tumor samples. These approaches will be developed as necessary.

Moreover, solving the problem of the efficient production of adrenal androgens by the organoids, as documented in Task 1, will be required before the influence of the organoids on breast cancer growth can be assessed.

### *Task 3*

Investigate the molecular biology of adrenal androgen regulation focusing on the key enzyme  $3\beta$ -hydroxysteroid dehydrogenase.

The initial approach for this task, as outlined in the grant application, was to overexpress the type II  $3\beta$ -HSD gene in human adrenal cells in order to prevent the production of DHEA and increase the production of cortisol. It now appears, from the data we have gathered, that this experiment is rendered unnecessary because we already have two types of human adrenal cells: one, the zona fasciculata cell, producing cortisol and essentially no DHEA, and the other, the zona reticularis cell, producing DHEA and essentially no cortisol (Endoh et al., 1996). However, as documented earlier, there appears to be a re-zonation of the cells when they are implanted in vivo. DHEA production, although high in reticularis cell cultures prior to implantation, is shut down during the development of the organoid. Therefore, we are changing our approach by engineering a cell in the reverse direction, that is, introducing an antisense  $3\beta$ -HSD construct to suppress the endogenous  $3\beta$ -HSD production and therefore, to increase DHEA production. Such an antisense approach requires that the cells to be used are capable of clonal growth and that the clonal cells are able to form an organoid when implanted in SCID mice. This is necessary because each transfection event (of an antisense construct) will create, in that particular cell and its progeny, some particular level of suppression of the targeted gene due to positional effects of integration of the transfected DNA and due to other unknown phenomena associated with antisense efficiency. Thus, the strategy of implanting a mass culture of many different transfected clones is unlikely to be

successful, because any cells which lack sufficient suppression by antisense would overwhelm the influence of those that have suppressed 3 $\beta$ -HSD. During the year, we have tested the ability of human and bovine adrenocortical cells when grown from single cells into clones to be able to produce functional organoids. We have found that human adrenal cells lack sufficient replicative potential for the production of organoids from implanted cell clones, but bovine adrenal cells, which are known to have a longer proliferative potential in culture, are able to produce functional organoids from a single clonal cell. The structure of such organoids by histology, immunocytochemistry and electron microscopy has been investigated and the production of cortisol has been documented in vivo and in vitro; in all respects they closely resemble organoids produced from primary bovine adrenal cells. As expected, the cells produce high amounts of cortisol, but not DHEA, as is characteristic for all bovine adrenal cells. However, we believe that suppression of the 3 $\beta$ -HSD in such bovine adrenal cells will produce a cell producing a high amount of DHEA, because we have already shown that pharmacological suppression of 3 $\beta$ -HSD in bovine adrenocortical cells in vitro is effective in causing them to synthesize large amounts of DHEA (Endoh et al., 1996). Antisense-expressing bovine adrenal cell implants would be predicted to produce very high levels of DHEA since they are essentially equivalent to a type of genetic deficiency of one of the enzymes. The decreased production of cortisol due to the antisense would increase the feedback via the hypothalamus and pituitary to stimulate the growth of the implanted cells, thus, incidentally increasing DHEA production, because DHEA does not exhibit feedback on the hypothalamo-pituitary axis. Consequently, the result is predicted to be a hyperfunctional and hyperplastic graft producing large amounts of DHEA similar conditions in the human condition of congenital adrenal hyperplasia. Experiments using the antisense approach are now being commenced.

#### *Task 4*

The physiological influences on adrenal androgen production in the human adrenal organoid/*scid* mouse model

This task awaits the development of adrenal organoids producing high amounts of DHEA as described in Tasks 1-3. We intend to commence this portion of the work when

such organoids have been developed.

*Task 5*

Identify the transcription factors which regulate the human type II 3 $\beta$ -HSD gene and test their effects on adrenal androgen synthesis in the human adrenal organoid/*scid* mouse model.

To address this task, we have taken advantage of our observation that DHEA production in the human adrenal cortex is exclusively the function of the zona reticularis rather than the zona fasciculata. Consequently, the regulation of the key gene causing this switch of steroidogenesis between DHEA and cortisol, namely, type II 3 $\beta$ -HSD gene, ought to be optimally investigated by a comparison of these two cell types. We have done this by separating the zones of the adult human adrenal cortex and preparing nuclear extract proteins from the zones. We have used these extracts to examine the distribution between the zones of transcription factors which regulate the type II 3 $\beta$ -HSD gene. To identify the region of the type II gene that is likely to be targeted by these factors, we took advantage of a previous observation that a 40-base pair region in the first intron of the type I 3 $\beta$ -HSD gene appears to be essential for regulation of this gene in tissues other than the adrenal cortex (Guerin et al., 1995). We also noted that this region differs significantly in the type II gene. Because the type II gene is expressed in the adrenal cortex, but not in other tissues, and the type I gene is expressed in other tissues, but not in the adrenal cortex, the presence of substantial nucleotide differences in this region makes it a likely region for the binding of transcription factors which are differentially regulated between the two genes and between the different tissues. Consequently, we made oligonucleotide probes for this region and used them in gel-shift assays to examine the potential differences between the zona fasciculata and zona reticularis. Such differences were indeed found. As shown in the Appendix, there is a reproducible difference in gel shift patterns between the zona fasciculata and zona reticularis extracts when using this DNA sequence as a probe. This indicates that one or more transcription factors binding to this region differ between the zones. Currently, we are using competitive gelshift analysis to identify the sequence that optimally binds the proteins that differ in distribution between the zones. We will then use such optimal binding sequences in experiments to identify and clone the factors. The

characterization of such factors would provide the first evidence for the nature of the molecular mechanisms by which the adrenal cortex forms functionally distinct zones, and thus would greatly assist in the task of engineering in vivo organoids which have appropriate zonation and which produce DHEA at the level of the normal human adrenal gland.

#### Conclusions

The essential feature of the *scid* mouse model for the regulation of human adrenal androgen biosynthesis and for the effects of these steroids on human breast cancer growth is the achievement of a functional zona reticularis cell in the implant. In order to make implants with these features, it is necessary to either restore normal zonation, by making an implant with a capillary bed suitable for this purpose, or to use genetically-modified cells with high DHEA secretion. In the future, characterization of the transcription factors regulating the type II 3 $\beta$ -HSD gene may also provide information on molecular regulation of zonation.

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Appendix

Demonstration of binding of zone-specific proteins to a regulatory region in the type I and II  $3\beta$ -HSD genes. HSD-1 = the fragment of the type I gene used; HSD-2 = the fragment of the type II gene used. Both fragments were end-labeled with  $^{32}\text{P}$  and were used in gelshift experiments using proteins derived from human adrenal zona fasciculata (ZF) or zona reticularis (ZR) or from a control cell line (HeLa). E, F, and G are bands representing proteins that differ between the zones.

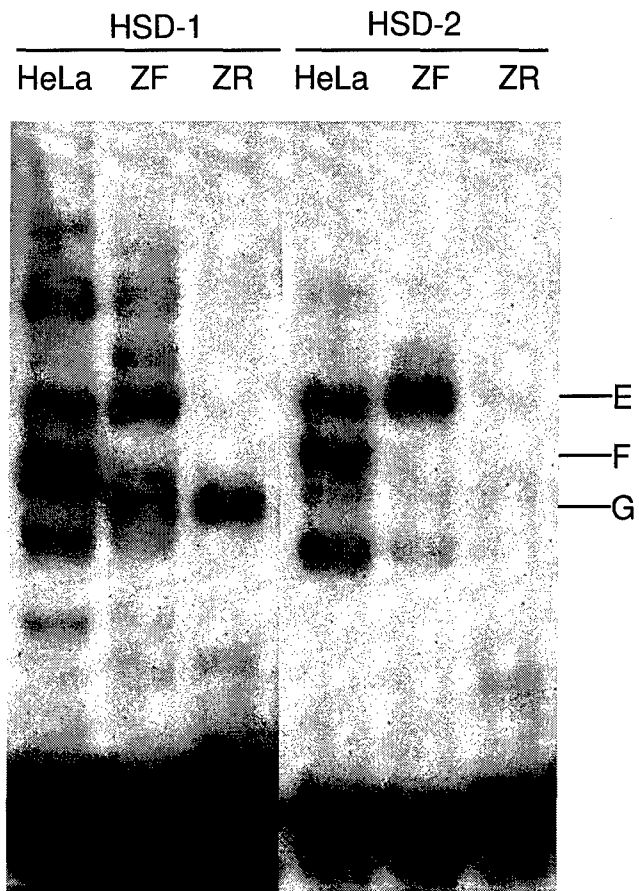
## HSD-1 and HSD-2 interact with zone-specific proteins of human adrenal

HSD - 1            GGACACAGAATGTTTGCAAAAAAAAAATGGGGTGGAGGAAAA  
HSD - 2            GGTCAATGGAATTTTGG - - TAAAAAATGGGGTGGAGGAAAA

Experiment:

Proteins: whole cell extracts of adrenal zonal cells

Probes : HSD-1 and HSD-2 oligos



1. Protein E, specific for HSD-2, is enriched in ZF cells;
2. Protein G, specific for HSD-1, is enriched in ZR cells.



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