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New methods for investigating experimental human adrenal tumorigenesis

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Abstract

Adenomas and nodules of the human adrenal cortex are common, whereas adrenocortical carcinomas are rare. Genes such as *IGF2* have been suggested to be important in human adrenocortical tumorigenesis but their role has not been directly investigated. We describe here elements of a system in which hypotheses concerning the molecular basis for the formation of benign and malignant adrenocortical lesions can be experimentally tested. Various viral vectors have been employed in the study of adrenocortical cell biology. Because of the low proliferative rate of primary human adrenocortical (pHAC) cells, a lentiviral system is ideal for transducing these cells with genes that may alter their characteristics or cause them to acquire benign or malignant tumorigenicity. Cultures of pHAC cells were highly infectible with lentiviruses and showed a higher proliferative potential when transduced with a lentivirus encoding *IGF2*. For tumorigenesis studies of genetically modified adrenocortical cells, we use *RAG2*^{-/-}, *γc*^{-/-} mice. Using this immunodeficient mouse model, we established an orthotopic intra-adrenal cell transplantation technique for adrenocortical cells that should be of value for future studies of the experimental conversion of human adrenocortical cells to a benign or malignant tumorigenic state.

Keywords

adrenal tumorigenesis; viral systems; immunodeficient mice; orthotopic cell transplantation

1. Introduction

Adenomas and nodules of the human adrenal cortex are common, whereas adrenocortical carcinomas are rare (Bornstein et al., 1999; Stratakis and Chrousos, 2000; Barlaskar and Hammer, 2007; Libe et al., 2007). In order to understand the pathogenesis of these adrenocortical lesions, a system is needed in which hypotheses concerning the molecular basis for the formation of nodules, adenomas and carcinomas can be experimentally tested. In this lab, we have worked toward the development of such an appropriate system. The major questions that we wish to address are (a) the experimental determination of the potential of various genes to confer on normal human adrenocortical cells the ability to form a range of

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tissue structures (corresponding to nodules, adenomas, and carcinomas) and (b) the potential for progression of cells from a benign to a malignant state, i.e. whether there is experimental evidence for the series nodule --> adenoma --> carcinoma in the adrenal cortex. Such progression is well established in other organs such as the colon (Vogelstein and Kinzler, 1993). Here we describe an experimental system in which we model human adrenocortical tumorigenesis by the use of primary human adrenocortical cells, viral vectors, and cell transplantation in immunodeficient mice.

In 2000, Hanahan and Weinberg proposed the concept of six classes of alterations in cell physiology, resulting from mutations or epigenetic changes, that would result in malignant growth: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, resistance to apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). How these principles apply to adrenocortical tumorigenesis is not yet clear; nodules and adenomas presumably have undergone fewer or different genetic changes than carcinomas. In this regard, microarray analysis to distinguish patterns of gene expression in different adrenal lesions is useful (Bornstein and Hornsby, 2005). One important gene that is more highly expressed in carcinomas is *IGF2* (Giordano et al., 2003; Slater et al., 2006). Data in experimental systems show an autocrine role for *IGF2* in metabolism, growth and differentiation of adrenocortical cells (Ilvesmaki et al., 1993; Weber et al., 1999). We show here the components of a complete experimental system for the study of the action of genes like *IGF2* in primary human adrenocortical cells and their effects in an in vivo setting, particularly with respect to orthotopic cell transplantation in immunodeficient mice.

2. Viral vectors for delivering potentially tumorigenic factors

Viral gene delivery systems have been tremendously valuable in elucidating molecular mechanisms involved in a wide variety of diseases as well as potentially valuable as therapeutic tools. There are three viral systems that have been employed: adenovirus, retrovirus and lentivirus.

2.1 Adenoviruses

There is evidence that the adrenal gland is a major target for adenoviruses; a selective tropism of this virus for the adrenal cortex has been demonstrated in experimental animals (Wang et al., 2003). Previously we transduced primary bovine adrenocortical cells with recombinant adenoviruses in several studies (Wolkersdorfer et al., 2002; Alesci et al., 2002; Chen and Hornsby, 2006). Our data showed that adenoviral-mediated gene transfer can interfere with the normal function of the adrenal cortex, leading to an impairment of adrenal steroidogenesis (Wolkersdorfer et al., 2002; Alesci et al., 2002). A single intra-adrenal injection of an adenoviral vector carrying the *CYP21* gene can compensate for the biochemical, endocrine, and histological alterations in 21-hydroxylase deficient mice (Tajima et al., 1999). As useful as these demonstrations are, adenoviral vectors do not fulfil the requirement of permanent genetic modification of adrenocortical cells. This leads us to consider the use of retroviruses and lentiviruses as vectors that can result in stable genetic modification of target cells.

2.2 Retroviruses

Unlike adenoviruses, retroviruses based on Moloney murine leukemia virus (MoMLV) integrate into the genome, leading to stable long-term expression. Previously, we showed that pBAC (primary bovine adrenocortical) and pHAC (primary human adrenocortical) cells co-transduced with retroviruses encoding Ras^{G12V} and SV40 T antigen can produce malignant tumors when transplanted in the subrenal capsule of immunodeficient mice. However, genetically modified pBAC cells were more aggressive in comparison to genetically modified

pHAC cells (Sun et al., 2004). This may suggest that the pHAC cells may require additional factors for tumorigenicity in comparison to pBAC cells. Retroviruses remain the main means of genetic modification for investigation of experimental conversion of adrenocortical cells to a malignant state. However, retroviral systems have the limitation that they can only infect dividing cells. Adult-derived pHAC cells divide slowly in culture (Hornsby and McAllister, 1991), thus making it difficult to obtain a high percentage of infected cells using retroviruses. Therefore it is desirable to employ a method for genetic modification that provides both long-term stable expression and that would be equally applicable to both dividing and nondividing cells.

2.3 Lentiviruses

Lentiviruses are retroviruses based on HIV-1 and similar viruses that can transduce both dividing and nondividing cells (Amado and Chen, 1999; Bartosch and Cosset, 2004). Like retroviruses based on MoMLV, lentiviruses in use for genetic modification are defective and unable to replicate in target cells. We tested the efficiency of a lentivirus encoding GFP in infection of pHAC cells. The transduction efficiency was very high (Figure 1). When pHAC cells were transduced with a lentivirus encoding IGF2 they exhibited a long-term stimulation of proliferation in comparison with cells that were transduced with a control lentivirus encoding GFP (Figure 2).

3. Immunodeficient host animal for cell transplantation studies

Another major concern for elucidation of the pathogenesis of benign and malignant lesions is the choice of an appropriate experimental model. The “gold standard” for testing the tumorigenic properties of genetically modified cells is cell transplantation in immunodeficient mice. For carcinomas, most types of immunodeficient mice can act as hosts for the growth of genetically modified human cells. There are also well-established surrogate cell culture assays for tumorigenicity, such as focus formation and anchorage-independent growth (Shin et al., 1975; Stiles et al., 1975). However, these methods are not equally applicable to experimental investigation of cells that may give rise to benign tumors as well as malignancies.

While *nude* and *scid* mice strains have been popular for testing tumorigenicity of human cells, they still retain natural killer (NK) cell activity that can affect the growth of transplanted genetically modified cells. In this lab over the last few years we have used a more profoundly immunodeficient mouse model, *RAG2*^{-/-}, *γc*^{-/-}, which lacks NK cell activity as well as T and B cells (Cooper et al., 2001). They serve as an excellent host for pBAC and pHAC cells that express Ras and SV40 T antigen, permitting the full expression of the malignant properties of these cells (Sun et al., 2004).

4. Sites in the body for transplantation of cells that may be tumorigenic

Cell transplantation provides a technique for the study of the physiology, cell biology, and molecular biology of adrenocortical cells in a three-dimensional vascularized tissue structure in a host animal. Cells survive, become vascularized by invasion of host endothelial cells, and secrete hormones into the circulation.

In experimental cell transplantation in immunodeficient mice, certain sites in the body and specific cell transplantation methods have been found to give superior results when investigating human cell tumorigenicity. In our studies on adrenocortical cells we have used both subrenal capsule cell transplantation and subcutaneous cell transplantation for various studies. Recently we began a study of the feasibility of orthotopic cell transplantation for adrenocortical cell studies and we report on preliminary observations on the use of this technique below.

4.1 Subcutaneous transplantation

In order to investigate the potential of cells for nodule, adenoma or carcinoma formation, a model is needed in which cells survive and function in a host animal independent of their potential to produce a malignant tumor. In conventional nude mouse tumorigenicity assays, the subcutaneous or intramuscular injection of cells that are not malignant causes them to die (Shin et al., 1975; Stiles et al., 1975). This is essentially how the assay is used; if the cells grow they are termed “tumorigenic” and if they do not grow, i.e. if they die, they are termed “nontumorigenic”. Thus, an assay in which cells are simply injected as a suspension under the skin of the mouse is unsuitable for studying cells that may not be aggressively malignant or may be normal or close to normal.

We first showed some time ago that normal pHAC and pBAC cells could survive and form a functional vascularized tissue structure when transplanted beneath the skin after being embedded in collagen gel (Popnikolov and Hornsby, 1999). This method proved useful for malignant as well as normal cells (Sun et al., 2004). It remains a valuable technique because tumor formation in this site is readily observable by noninvasive techniques (Sun et al., 2004).

4.2 Subrenal capsule transplantation

The subrenal capsule site is an excellent site for the growth of normal and abnormal adrenocortical cells. In accordance with their genetic modifications, adrenocortical cells transplanted under the kidney capsule of immunodeficient mice can produce (i) normal functional vascularized tissue, or (ii) a benign (expanding, non-invasive) adenoma-like structure, or (iii) a malignant (invasive and metastizing) tumor. Thus, the model provides a way to investigate what genetic changes can convert normal adrenocortical cells into a benign, as well as a malignant, lesion, or produce a tissue structure with no detectable abnormalities (Thomas et al., 1997; Thomas et al., 2000; Thomas et al., 2002; Thomas et al., 2003; Huang et al., 2007). However, as excellent as this site is, it is still ectopic for adrenocortical cells, and therefore we recently turned to orthotopic cell transplantation to address this issue.

4.3 Orthotopic cell transplantation

Orthotopic transplantation models are important for a complete understanding of tumor biology (Togo et al., 1995; Singh et al., 1997; Killion et al., 1998). The properties of malignant and nonmalignant cells depend on their local microenvironment. In the environment of their normal host tissue, the behavior of cells may differ considerably from that in ectopic sites.

Orthotopic cell transplantation has not been reported for the adrenal cortex. However, neuroblastoma cells have been transplanted into the mouse adrenal gland. These studies showed superior rates of tumor growth, vascularization, and metastasis in comparison to ectopically transplanted cells (Khanna et al., 2002; Henriksson et al., 2004; Joseph et al., 2005).

We therefore began preliminary experiments to test whether orthotopic transplantation was feasible for cells of adrenocortical origin. We established an orthotopic transplantation method using pBAC cells transduced with retroviruses encoding Ras/SV40 T antigen/hTERT/GFP (Sun et al., 2004), by injection of the cells into the adrenal glands of RAG2^{-/-}, γ c^{-/-} mice (Figure 3). The results of these preliminary studies show that orthotopic cell transplantation by in the mouse adrenal gland is technically feasible, as SV40 T antigen-expressing cells were found within the gland and expanding beyond the gland itself.

5. Conclusion

In summary, a suite of methods is in place that allows us to investigate the genetic modifications that may convert primary human or bovine adrenocortical cells to a fully malignant state or to various forms of benign lesions with properties of nodules or adenomas. Lentiviral transduction of pHAC cells should provide an efficient method for genetic modification of cells without excessive cell division. Orthotopic cell transplantation provides an appropriate in vivo microenvironment for the growth of genetically modified cells into a malignant or benign tissue structure. The combination of primary culture of human adrenocortical cells, lentiviral modification, and rapid orthotopic transplantation of the cells provides an excellent system for studying the genetic changes that human adrenocortical cells must acquire to exhibit various stages of malignant behavior. In the future this system could also be used to help to design therapeutic strategies for human adrenal cancer.

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Figure 1.

Primary human adrenocortical cells can be efficiently transduced with lentiviruses.

Adrenocortical cells obtained from a 47 year-old male donor were cultured using conditions previously established for these cells (Hornsby and McAllister, 1991) with the exception that collagen-coated dishes were used rather than regular tissue culture plastic. A lentivirus encoding enhanced green fluorescent protein, pWPXL-GFP, was produced using conventional methods in 293T cells (Pear, 1996). One week after plating the cells in culture, unconcentrated virus was added to the cells in the presence of 20 $\mu\text{g/ml}$ Polybrene for 3 hours. The efficiency of infection was monitored using fluorescence microscopy; the images above show cells photographed by fluorescence and by phase-contrast at 72 hours after infection.

Figure 2.

In vitro effects of lentivirally delivered IGF2. Adrenocortical cells obtained from a 67 year-old female donor were cultured as described in the legend to Figure 1. Cells were infected with a lentivirus encoding IGF2 (pReceiver-Lv01-IGF2 from Genecopoeia Inc.) or with a control GFP lentivirus. Following infection, cells were grown with subcultures at 10-day intervals for 30 days. After this period of time in culture, relatively few cells remained in the control culture of cells expressing GFP (a) whereas continued survival and proliferation is evident in the culture of cells expressing IGF2 (b).

Figure 3.

Orthotopic adrenocortical cell transplantation. Primary bovine adrenocortical (pBAC) cells were co-infected with two retroviruses (one encoding Ras, SV40 T antigen and GFP and a second encoding hTERT; Sun et al., 2004). All cells prior to transplantation were positive for expression of both SV40 T antigen and GFP. Cells (5×10^5) were embedded in a mixture of thrombin and fibrinogen (Fingert et al., 1987) and injected into the adrenal gland of female immunodeficient mice (*RAG2^{-/-}, γ c^{-/-}*) using a 30 gauge needle. Nineteen days after transplantation mice were sacrificed. (a) In situ evaluation of the adrenal gland and surrounding tissues following transplantation of genetically-modified pBAC cells. Expansion of the transplanted cell population was visualized by fluorescence using a 470 nm light source. Following this photography, tissues were fixed, embedded in paraffin and sectioned for histological analysis. (b) Hematoxylin and eosin staining of tissue formed from genetically modified pBAC cells. Asterisk indicates region of remaining mouse adrenocortical tissue. (c) Demonstration of localization of genetically modified cells using immunohistochemistry with

anti-SV40 T antigen (monoclonal antibody PAb416). (d) Immunohistochemistry using a polyclonal antibody against cholesterol side-chain cleavage enzyme (Calbiochem Inc.) shows remaining mouse adrenocortical cells, particularly in area indicated by asterisk.