Molecular and Cellular Endocrinology 436 (2016) 250–258

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Transplantation of alginate-encapsulated seminiferous tubules and interstitial tissue into adult rats: Leydig stem cell differentiation in vivo?

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ARTICLE INFO

Article history:
Received 10 May 2016
Received in revised form
9 August 2016
Accepted 29 August 2016
Available online 31 August 2016

Keywords:
Stem cells
Leydig cells
Alginate
Transplantation
Testosterone

ABSTRACT

In vivo and in vitro studies were conducted to determine whether testosterone-producing Leydig cells are able to develop from cells associated with rat seminiferous tubules, interstitium, or both. Adult rat seminiferous tubules and interstitium were isolated, encapsulated separately in alginate, and implanted subcutaneously into castrated rats. With implanted tubules, serum testosterone increased through two months. Tubules removed from the implanted rats and incubated with LH produced testosterone, and cells on the tubule surfaces expressed steroidogenic enzymes. With implanted interstitial tissue, serum levels of testosterone remained undetectable. However, co-culture of interstitium plus tubules in vitro resulted in the formation of Leydig cells by both compartments. These results indicate that seminiferous tubules contain both cellular and paracrine factors necessary for the differentiation of Leydig cells, and that the interstitial compartment contains precursor cells capable of forming testosterone-producing Leydig cells but requires stimulation by paracrine factors from the seminiferous tubules to do so.

1. Introduction

Testosterone, produced by Leydig cells of the mammalian testis, plays an essential role in the development and maintenance of the male reproductive system, as well as in metabolism, muscle mass, and bone mineral density (Mooradian et al., 1987; Tuck and Francis, 2009). Previous studies have shown that in both the human and rat, testosterone formation gradually increases from the peripubertal period through the adult, coincident with the development of adult Leydig cells (Habert et al., 2001; Svechnikov et al., 2010; Teerds and Huhtaniemi, 2015). In mice and rats, the adult Leydig cells develop from stem cells (stem Leydig cells, referred to herein as SLCs) which, in postnatal day 7 testes, express the stem cell markers nestin (Davidoff et al., 2004; Jiang et al., 2014), COUP-TFII (Qin et al., 2008; Kilcoyne et al., 2014), Arx (Miyabayashi et al., 2013), CD51 (Jiang et al., 2014), p75NTR (Jiang et al., 2014), and platelet-derived growth factor receptor PDGFRα (Ge et al., 2006; Landreh et al., 2013). These cells do not express Leydig cell lineage markers (Ge et al., 2006; Landreh et al., 2013; Davidoff et al., 2004; Jiang et al., 2014; Kilcoyne et al., 2014). By day 11 postpartum, some of the SLCs commit to a differentiation pathway, forming progenitor Leydig cells (PLCs) that express the Leydig cell lineage markers 3β-hydroxysteroid dehydrogenase (3βHSD), cholesterol side-chain cleavage (P450scc or CYP11A1) and luteinizing hormone receptor (Benton et al., 1995; Chen et al., 2010). The PLCs differentiate into immature Leydig cells (ILCs) from day 21 to day 35, and the latter into adult Leydig cells (ALCs) from day 28–56 (Benton et al., 1995; Chen et al., 2009, 2010; Teerds and Huhtaniemi, 2015).

Numerous studies have shown that the elimination of the Leydig cells from the adult rat testis by treating rats with the alkylating agent ethane dimethanesulfonate (EDS) is followed by the formation of a new generation of ALCs (Jackson et al., 1986; Kerr et al., 1987). The new cells arise from stem cells that proliferate and then differentiate (Jackson et al., 1986; Kerr et al., 1987; Davidoff et al., 2004; Stanley et al., 2012; Li et al., 2016). The location(s) of

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http://dx.doi.org/10.1016/j.mce.2016.08.046
0303-7207/

the stem cells, and the nature and origin of regulatory factors involved in their proliferation and differentiation to ALCs, remain uncertain (Davidoff et al., 2004; O'Shaughnessy et al., 2008; Chen et al., 2010; Stanley et al., 2012; Li et al., 2016).

Recently, we reported that PDGFRα-expressing cells isolated from the testes of EDS-treated rats had the ability to proliferate for extended periods of time in vitro, or to differentiate into testosterone-producing cells (Stanley et al., 2012). These are properties expected of stem cells. When isolated rat seminiferous tubules were cultured in vitro, functional Leydig cells were generated on their surfaces (Stanley et al., 2012; Zhang et al., 2013; Odeh et al., 2014; Li et al., 2016). However, under similar culture conditions, the interstitium failed to form functional Leydig cells (Stanley et al., 2012), indicating that the interstitium may lack stem cells, regulatory (niche) factors, or both. Previous studies, however, provided evidence that various cells of the testicular interstitium might be precursors of Leydig cells, including peritubular myoid cells (O'Shaughnessy et al., 2008; Stanley et al., 2012), blood vessel-associated pericytes (Davidoff et al., 2004), mesenchymal cells (Hardy et al., 1989), or combinations of fibroblasts, lymphatic endothelial cells and pericytes (Jackson et al., 1986).

In the present study, we pursued an in vivo approach to ask whether testosterone-producing Leydig cells would be generated from rat seminiferous tubules and/or from interstitial tissue in the absence of paracrine influences of one on the other. A number of previous studies in the rodent, dog, and human had shown that encapsulation in alginate-poly-L-lysine can effectively maintain the integrity of implanted tissue and cells and prevent immunorejection (Barsoum et al., 2003; de Vos et al., 2006; Orive et al., 2006). This approach has been used for applications that include insulin delivery by pancreatic islet cells, therapeutic gene product delivery by recombinant cells, and testosterone delivery by adult Leydig cells (Barsoum et al., 2003; Machluf et al., 2003; de Vos et al., 2006). Using alginate encapsulated seminiferous tubules and interstitium, we show in the present studies that: 1) cells associated with seminiferous tubules are able to develop into testosterone-producing Leydig cells in vivo outside the testis and therefore in the absence of paracrine factors from the interstitial compartment; 2) in contrast, interstitial tissue failed to form functional Leydig cells in vivo; but 3) in vitro co-culture of interstitium with seminiferous tubules resulted in the formation of testosterone-producing cells by the interstitium, We conclude that the interstitial compartment contains Leydig cell precursor cells, but lacks required paracrine factors from the seminiferous tubules to differentiate into testosterone-producing Leydig cells.

2. Materials and methods

2.1. Chemicals

Tissue culture supplements ITS (insulin/transferrin/selenite), fetal bovine serum (FBS), sodium alginate, alginate lyase, poly-L-lysine (21 kDa), and β-actin antibody were from (Sigma-Aldrich) (St. Louis, MO). M199 and DMEM/F12 medium and Hank's Balanced Salt Solution (HBSS) were from Invitrogen (Carlsbad, CA). [1,2,6,7,16,17-^{3}H(N)]-Testosterone (115.3 Ci/mmol) was from Perkin Elmer Life Sciences, Inc (Boston, MA). Testosterone antibody was from ICN (Costa Mesa, CA). CYP11A1 antibody was from Chemicon International (Temecula, CA). Bovine LH (USDA-bLH-B-6) was provided by the USDA Animal Hormone Program (Beltsville, MD). Testosterone was from Steraloids (Newport, RI). Reagents for morphological studies were from Electron Microscopy Sciences (Hatfield, PA).

2.2. Animals

Adult male Sprague-Dawley rats (3 month-old) were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN). Rats were housed in the animal facilities of the Johns Hopkins School of Public Health, under conditions of controlled light (14 h light: 10 h dark) and temperature (22 °C) and with free access to rat chow and water. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, according to protocols approved by the Johns Hopkins Animal Care and Use Committee.

2.3. Isolation and encapsulation of seminiferous tubules and interstitial tissue

Rats received a single, intraperitoneal injection of ethane dimethanesulfonate (EDS; 85 mg/kg BW) to eliminate Leydig cells, and were sacrificed by decapitation 4 days later. The testes were perfused with DMEM/F12 medium via the testicular artery to eliminate blood. After decapsulation, testes were separated into 8 equal-sized pieces. The seminiferous tubules were mechanically separated from the interstitium using fine forceps (Toppari et al., 1991; Stanley et al., 2012). Tubules and interstitium were encapsulated separately in alginate gel as reported previously (Machluf et al., 2003), but with minor modifications as follows: Tubules or interstitial tissue were placed briefly into 1.2% sodium alginate in Ca^{2+}- and Mg^{2+}-free HBSS solution, and then incubated (5 min) in calcium solution (1.5% $CaCl_2$ in HBSS) to crosslink the alginate. After washing with HBSS to remove free calcium ions, the capsules were coated with 0.1% poly-L-lysine in HBSS (12 min). Unreacted poly-L-lysine was removed by washing the capsules 3 times with HBSS, and the capsules were additionally coated with 0.125% sodium alginate in HBSS (10 min). The encapsulated tissues were either cultured in vitro or transplanted into animals. For in vitro studies, 5 cm lengths of tubules were encapsulated in alginate and cultured in 24 well plates. For in vivo experiments, multiple tubule fragments were encapsulated together. A total of 8 capsules of tubules and of interstitium were prepared from each testis.

2.4. Culture of seminiferous tubules and interstitium in vitro

Dual-chamber co-culture experiments were conducted. Seminiferous tubules (10 cm total length per testis) were incubated in the inner chamber of the Millicell Hanging Inserts (6 well, 1.0 μm pore; MilliporeSigma, Billerica, MA). Interstitium (isolated from 1/8 of each testis) was placed in the outer chamber. Previous studies showed that proliferation of SLCs associated with rat seminiferous tubules occurs when the tubules are cultured with LH for one week, and that differentiation occurs during weeks 2–3 (Odeh et al., 2014; Li et al., 2016). To examine the effects of co-culture on proliferation, interstitium and tubules were cultured together in the presence of LH in the medium during week 1, and then were separated and cultured with LH alone for 3 additional weeks. To examine the effects of co-culture on differentiation, the interstitium was cultured with LH for the first week, co-cultured with tubules in the presence of LH for 2 additional weeks, and then separated from the tubules and cultured alone for another week with LH. To assess the ability of the interstitium to produce testosterone after these treatments, tissue was incubated with maximally stimulating LH (10 ng/ml) for 24 h, and testosterone was assayed in the medium. Additionally, interstitial cells were stained for 3β-HSD after dispersed the cells with collagenase (1 mg/ml; 20 min) and attached to culture plate for 24 h (see section 2.6).