Reproductive Toxicology 50 (2014) 68–86

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Altered gene expression patterns during the initiation and promotion stages of neonatally diethylstilbestrol-induced hyperplasia/dysplasia/neoplasia in the hamster uterus

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ARTICLE INFO

Article history:
Received 19 April 2014
Received in revised form 18 August 2014
Accepted 8 September 2014
Available online 19 September 2014

Keywords:
Female reproductive system
Uterus
Diethylstilbestrol
Endocrine disruption
Neoplasia

ABSTRACT

Neonatal treatment of hamsters with diethylstilbestrol (DES) induces uterine hyperplasia/dysplasia/neoplasia (endometrial adenocarcinoma) in adult animals. We subsequently determined that the neonatal DES exposure event directly and permanently disrupts the developing hamster uterus (initiation stage) so that it responds abnormally when it is stimulated with estrogen in adulthood (promotion stage). To identify candidate molecular elements involved in progression of the disruption/neoplastic process, we performed: (1) immunoblot analyses and (2) microarray profiling (Affymetrix Gene Chip System) on sets of uterine protein and RNA extracts, respectively, and (3) immunohistochemical analysis on uterine sections; all from both initiation stage and promotion stage groups of animals. Here we report that: (1) progression of the neonatal DES-induced hyperplasia/dysplasia/neoplasia phenomenon in the hamster uterus involves a wide spectrum of specific gene expression alterations and (2) the gene products involved and their manner of altered expression differ dramatically during the initiation vs. promotion stages of the phenomenon.

1. Introduction

The medical misadventure commonly known as the "DES Syndrome" resulted from the mistaken belief that treatment during pregnancy with diethylstilbestrol (DES), the first orally active estrogen [1], would protect against miscarriage [2]. That treatment regimen began in 1947 and then quickly and greatly expanded worldwide [2] even though evidence questioning its effectiveness appeared as early as 1953 [3]. Unfortunately, it was not until 1971 with two independent reports of clear cell vaginal adenocarcinoma in the young daughters of DES-treated mothers that such treatment ceased [2]. Since then, numerous clinical and experimental animal studies of the effects of perinatal DES exposure documented teratogenic and neoplastic lesions throughout both the female and male reproductive tracts and thereby established DES as a transplacental carcinogen and the prototypical endocrine disruptor agent [2,4].

To study the phenomenon of perinatal DES-induced endocrine disruption, we established a convenient and sensitive model system using Syrian golden hamsters [5]. In that system, we defined the progression and extent of endocrine alterations and morphological lesions in the reproductive tracts of both females and

Abbreviations: ABC, avidin–biotin–horseradish peroxidase complex; AR, androgen receptor; CON, control; DAB, diaminobenzidine; DES, diethylstilbestrol; E2, estradiol-17β; EMT, epithelial/mesenchymal transition; ERα, estrogen receptor alpha; ECM, extracellular matrix; FDR, false discovery rate; H&E, hematoxylin and eosin; IHC, immunohistochemistry; IRF-1, interferon regulatory factor 1; IRS-1, insulin receptor substrate 1; IS, initiation stage; Keap, Kelch-like ECH-associated protein; LEF, lymphoid enhancer factor; NES1, normal epithelial-cell specific 1; NFκB, nuclear factor kappa B; Nrf2, nuclear factor (erythroid 2-related) factor 2; NSB, non-specific bands; O, ovariectomized; PBS, phosphate-buffered saline; PBST, PBS plus 0.05% Tween 20; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor; PS, promotion stage; PTM, PBST plus 5% nonfat dry milk; SAFB1, scaffold attachment factor B1; s.c., subcutaneous; Sp1, specificity protein 1; Stat5A, signal transducer and activator of transcription 5a; TCF, T cell factor; WB, Western blot/blotting.

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http://dx.doi.org/10.1016/j.reprotox.2014.09.002
0890-6238/

males [5–9]. A particularly striking observation very early in the system was that, in mature (postpubertal) hamsters, 100% of the neonatally DES-exposed uteri developed hyperplasia and a large proportion progressed to neoplasia (endometrial adenocarcinoma) [5]. We subsequently determined that, consistent with the two-stage model of carcinogenesis [10], neonatal DES exposure directly and permanently alters (re-programs) the developing hamster uterus (initiating event) such that it responds abnormally later in life to stimulation (promoting event) with the natural estrogen, estradiol [5,6]. We are now probing the mechanism of this two-stage phenomenon at the molecular level. Here we report that: (1) progression of the neonatal DES-induced hyperplasia/dysplasia/neoplasia phenomenon in the hamster uterus involves a wide spectrum of specific gene expression alterations and (2) the gene products involved and their manner of altered expression differ dramatically during the initiation vs. promotion stages of the phenomenon.

2. Materials and methods

2.1. General animal information

Animals were maintained and treated in an AAALAC-accredited facility as authorized by the Wichita State University Institutional Animal Care and Use Committee (IACUC). All procedures including neonatal treatment, anesthesia, ovariectomy, chronic estrogenic stimulation, sacrificing, and tissue collections followed well-established [5–7] and IACUC-approved protocols.

2.2. Neonatal animal treatment

Timed pregnant Syrian golden hamsters (*Mesocricetus auratus*) from Charles River Breeding Laboratories (Wilmington, MA) or Harlan Sprague Dawley, Inc. (Indianapolis, IN) were caged singly under a 14 h light:10 h dark photoperiod at 23–25 °C with laboratory chow and water provided ad libitum. The food was a 2:1 mixture of #5001 rodent diet and #5015 mouse diet from LabDiet (PMI Nutrition Int. LLC, Brentwood, MO). According to the manufacturer, total isoflavone (diadzein, genistein, glycitein) content of that diet mixture was 426 mg/kg. Within 6 h of birth (day 0), litter size was adjusted to eight neonates/litter by eliminating males and all animals in a litter received a single s.c. injection of 50 μl corn oil vehicle either alone (control, CON) or containing 100 μg of DES (both from Sigma Chem. Co., St. Louis, MO). As acknowledged previously [5–7], that dose level is high but not unreasonable considering that DES ingestion levels by women were as much as 150 mg daily and 18.2 g total during their pregnancy [11], with the median total dose being 10.7 g [12]. It is also the dose level we previously used in hamsters to establish, assess, and compare neonatal DES-induced disruption in various regions of both the female and male reproductive tract [5–7]. Tissues were harvested (see below) from some of these neonatal animals when they were 5 days of age (initiation stage or IS).

2.3. Prepubertal procedures

On day 21 of life (~7 days prior to puberty), groups of control and neonatally DES-treated animals were bilaterally ovariectomized and began chronic estrogen stimulation by the s.c. insertion (between the shoulder blades) of a plugged Silastic (Dow Corning Corp., Midland, MI) tube (open lumen length, 1.0 cm; inner diameter, 1.57 mm; outer diameter, 2.41 mm) filled with crystalline estradiol-17β (from Sigma Chem. Co.; St. Louis, MO) (O + E2). According to previous determinations [5,6], that procedure maintains serum E2 levels at approximately 200 pg/ml for at least 5 mo. Tissues were harvested (see below) from the O + E2 animals when they were 2 months of age (promotion stage or PS).

2.4. Tissue harvesting and processing

Both IS and PS animals were anesthetized/asphyxiated with CO_2 and then decapitated. For preparation of total protein and RNA extracts, isolated and trimmed uterine horns were snap frozen on dry ice and cryostored at −80 °C. For histological processing, mid-region uterine horn segments from freshly killed animals were immediately placed in fixative (4% paraformaldehyde in Dulbecco's phosphate-buffered saline [PBS], pH 7.2) followed by two changes (24 h each) of fresh fixative, stored in 70% ethanol, and ultimately embedded in paraffin so as to generate transverse (cross) sections.

2.5. Preparation of total protein extracts

Frozen tissues were quickly weighed, received 9 volumes (ml/g) of hypotonic buffer (10 mM Tris base, 1 mM ethylenediaminetetraacetic acid, pH 7.5), homogenized on ice (2× 5 s with a Tekmar Model TR-19 Tissuemizer at a power setting of 70), received ¼ volume of 5× sample buffer (to provide a final concentration of 0.1 M dithiothreitol, 2% sodium dodecyl sulphate, 80 mM Tris base, pH 6.8, 10% glycerol, 1% saturated bromophenol blue solution), brought to 100 °C for 5 min, and cryostored at −20 °C. As noted previously [8], this procedure yields groups of denatured total protein extracts that are matched or normalized based on tissue equivalents.

2.6. Preparation of total RNA extracts

Frozen tissues (50–100 mg) received 1 ml TRIzol® Reagent (Ambion, Grand Island, NY), were homogenized as above (preparation of protein extracts), incubated at 25 °C for 5 min, received 0.2 ml chloroform and mixed (shaken by hand for 15 s), incubated at 25 °C for 2–3 min, and then centrifuged (12,000 × *g* at 4 °C for 15 min). The resulting upper/aqueous phase fractions were transferred to pre-centrifuged Eppendorf Phase Lock Gel tubes, re-mixed, and re-centrifuged. The resulting upper/aqueous phase fractions were transferred to fresh tubes, received 0.5 ml isopropanol, incubated at 25 °C for 10 min, re-centrifuged, supernatants were aspirated, RNA pellets were air-dried, dissolved in 100 μl RNase-free water at 55–60 °C for 10 min, and stored at −80 °C following spectrometric analysis/quantitation at 230/260/280/320 nm.

2.7. Western blot analysis

The Western blot (WB) procedure was as fully described for our analysis of extracts prepared from male hamster reproductive tract tissues [8]. In brief, sets of uterine extract aliquots (25 μl) prepared from control and neonatally DES-exposed animals (both IS and PS) were electrophoresed under denaturing conditions on 5–15% acrylamide gradient gels. The gels were either Coomassie stained to visualize the overall pattern of resolved proteins or were electro-transferred to nitrocellulose membranes. The membrane blots were probed with antibodies directed against the indicated protein targets (see Table 1 for descriptions of antibody provider, species source, monoclonal or polyclonal type, and dilution or concentration used) and the primary antibody:antigen complexes were detected using a biotin-labeled and species-specific anti-IgG second antibody followed by an avidin–biotin–horseradish peroxidase complex (ABC) reagent (both from Vector Laboratories, Burlingame, CA) and finally a diaminobenzidine (DAB) substrate reagent (SigmaFast™ from Sigma, St. Louis, MO) that generates deposition of an insoluble, dark-brown product. Densitometric scans of the blots were analyzed using Quantity One® quantitation