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Adipocyte derived paracrine mediators of mammary ductal morphogenesis controlled by retinoic acid receptors ☆

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ABSTRACT

We generated a transgenic (Tg)-mouse model expressing a dominant negative-(DN)-RARα, (RARαG303E) under adipocytes-specific promoter to explore the paracrine role of adipocyte retinoic acid receptors (RARs) in mammary morphogenesis. Transgenic adipocytes had reduced level of RARα, β and γ, which coincided with a severely underdeveloped pubertal and mature ductal tree with profoundly decreased epithelial cell proliferation. Transplantation experiments of mammary epithelium and of whole mammary glands implicated a fat-pad dependent paracrine mechanism in the stunted phenotype of the epithelial ductal tree. Co-cultures of primary adipocytes, or *in vitro* differentiated adipocyte cell line, with mammary epithelium showed that when activated, adipocyte-RARs contribute to generation of secreted proliferative and pro-migratory factors. Gene expression microarrays revealed a large number of genes regulated by adipocyte-RARs. Among them, pleiotrophin (PTN) was identified as the paracrine effectors of epithelial cell migration. Its expression was found to be strongly inhibited by DN-RARα, an inhibition relieved by pharmacological doses of all-trans retinoic acid (atRA) in culture and *in vivo*. Moreover, adipocyte-PTHR, another atRA responsive gene, was found to be an up-stream regulator of PTN. Overall, these results support the existence of a novel paracrine loop controlled by adipocyte-RAR that regulates the mammary ductal tree morphogenesis.

Introduction

Retinoids and their receptors (RARs and RXRs) have been extensively studied in embryonic morphogenesis, but their role in post-natal tissue development has received sparse attention, partly because of the embryonic or peri-natal lethality of compound RAR mutants. The mammary gland is uniquely suited for post-natal study as a major morphogenic expansion occurs during the 2nd month after birth. One of several transgenic approaches we used to modulate RAR function was the expression, in mouse mammary epithelium, of a full length RARα with a point mutation in G303E which functions as a dominant negative (DN)-RARα. This approach produced rapidly developing B cell lymphoma in these mice (Wang et al., 2005); (Kupumbati et al., 2006), precluding a detailed examination of the RAR in normal mammary and tumors. Retinoic acid treatment slowed the growth of lymphomas suggesting that the construct functions as a DN-RARα (Wang et al., 2005).

Because adipocytes have been shown to affect mammary epithelial function, and since RARs, potent transcriptional regulators, are expressed in adipose tissue (Alvarez et al., 2000), including the mammary fat pad, we wondered whether they might influence mammary morphogenesis. Under the influence of pubertal hormones, the rudimentary mammary epithelial tree present at birth, begins extending and invading the mammary fat pad. Elongation and branching of the ducts, regulated by proliferation and migration of terminal end buds (TEB) cells, rely on both endocrine and local growth regulatory signals, ECM remodeling and stromal–epithelial interactions. Because the leading edge of the TEB is mostly devoid of myoepithelial layer and has fragmented basement membrane, it is directly juxtaposed to the surrounding fat-pad adipocytes. The mammary fat pad is made mainly of white adipocytes, although some brown adipocytes have been identified (Gouon-Evans and Pollard, 2002). We propose that the directional ductal epithelial elongation might be influenced by the TEB contact with, and duct proximity to, the fat pad. To date, little is known about molecular interactions between these two compartments. *In vitro*, proliferation of mammary epithelium was shown to increase in the presence of mammary fat-pad explants or ECM and conditioned medium derived from 3T3-L1 adipocytes (Hovey et al., 1998; Levine and Stockdale, 1984; Rahimi et al., 1994). Primary mammary epithelium undergoes ductal morphogenesis when cultured on a monolayer of 3T3-L1 adipocytes or when co-cultured with fetal mammary fat-pad precursor tissue (Kanazawa and Hosick, 1992; Wiens et al., 1987). Mammary adipocytes also induce alveolar morphogenesis and enhance functional differentiation of epithelial cells in a Transwell co-culture

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model (Darcy et al., 2000; Zangani et al., 1999). The virtual lack of ductal development in A-ZIP/F-1 fatless mouse (Couldrey et al., 2002) most convincingly demonstrates that adipocytes play an indispensable role in mammary development. Although, these studies demonstrate the importance of adipocytes in the development of mammary epithelial cells, they provide no clues to the mechanisms involved.

To analyze the molecular events that might be responsible for the adipocyte effects, we generated transgenic mice in which adipocytes-RAR function was inhibited by the expression of G303E point mutant of RARα. This dominant negative (DN) mutation in the ligand binding domain of RAR, which precludes the binding of physiological concentrations of ligand (Kupumbati et al., 2006; Saitou et al., 1994) gives rise to a severely stunted ductal mammary tree. A microarray analysis showed that the presence of DN-RARα modulated the expression of a large number of genes, among them growth factors, proteins mediating cell adhesion and migration and proteins previously shown to modulate mammary development. We focused on 2 of these proteins, a membrane receptor PTHR and pleiotrophin, a secreted protein, both down-regulated in the transgenic glands and showing interconnected regulation and paracrine effects on mammary epithelium. Overall, our results established adipocyte-RARs as indispensable regulators of a secreted growth factor (pleiotrophin), involved in paracrine regulation of epithelial ductal tree development.

Materials and methods

Generation of aP2.RARαG303E mice

A blunt ended RARαG303E fragment (Saitou et al., 1994) was cloned into Sma 1 digested PBks-AP2 vector (a gift from Dr. Bruce Spiegelman) (Graves et al., 1992). The fragment obtained by Sal 1 and Not 1 digestion and purified by agarose gel was used for microinjection. Three founders (lines 8, 10 and 16) were generated in FVB/N background by standard pronuclear injection. Southern blot was used to determine amounts of transgene DNA relative to wt-RARα DNA using methods and reagents as previously described (Kupumbati et al., 2006). Hemizygous lines were established from all 3 founders. We found that the number of viable pups was somewhat greater when transgenic males were mated with wild type females; hence this approach was used for long term line maintenance. The Tg- females did not nurse their pups. PCR-based genotyping was routinely used. All 3 lines exhibited similar phenotypes and lines 8 and 16 were continuously maintained. Transgene expression was monitored by RT-PCR using the HA tag.

Mammary glands whole mounts

Mammary glands were excised, fixed in Carnoy's fixative and stained in Carmine Alum Solution. For details: http://mammary.nih.gov/tools/histological/Histology/index.html.

Ductal tree proliferation assay

To compare proliferation of cells in the ductal mammary tree of the wt and aP2.RARaG303E mice, the Click-It EdU Alexa Fluor 488 Imaging Kit from Invitrogen was used. Briefly, 8-week old female mice were injected with 100 μg EdU (5-ethynyl-2′-deoxyuridine) in 100 μl of PBS 3 h prior to gland removal, the glands were fixed in 10% formalin in PBS, embedded in paraffin and 5 μm sections were prepared and processed for staining as per manufacturer instructions. Alexa-488 green fluorescent nuclei (proliferating cells) were visualized under the fluorescence microscope and quantified by calculating the % of Alexa-488 positive nuclei per 300 DAPI positive epithelial cells at 200× magnification. Six wt and 6 Tg glands were used for quantification.

Epithelial and whole gland transplantation

Epithelium containing fragments of mammary glands of 6-week old virgin mice (wt or Tg) taken from the area between the nipple and the LN, were transplanted into epithelium pre-cleared glands as previously described (Kupumbati et al., 2006). The recipient glands were processed for whole mounts 8 weeks after transplantation. In total 24 epithelial fragments were transplanted; 7 mice were mock transplanted to ascertain the efficiency of epithelial removal. For whole gland transplantation, gland number 4 from a 4-week old wt or Tg-virgin mice were transplanted under the skin of 6-week old recipient, by placing the transplant under the skin between gland number 3 and 4. The glands were excised and examined as whole mounts 8 weeks after transplantation. In total 6 pairs of glands were transplanted.

Adipocyte isolation

Adipocytes of 7-week old virgin mice were prepared from left number 4 gland (#4L), and both number 3 glands (#3L/R); the number 4 right gland (#4R) was kept for whole mount analysis. Glands were digested in collagenase/BSA solution at 37 °C for 2 h and centrifuged for 10 min at 900 rpm, the floating pellet containing adipocytes was removed, examined under microscope for purity, stabilized with RNAlater solution (Ambion), and stored at −20 °C until RNA isolation.

Induction of adipocyte differentiation in 3T3-L1 murine preadipocyte fibroblast cell line

3T3-L1 cells (a gift from Dr. Philip Scherer's Lab, Albert Einstein School of Medicine, NY) were propagated at 50–60% confluence in DMEM with 10% FBS (Benchmark FBS, Hyclone Laboratories, Inc) and 1× Penicillin–Streptomycin solution. For differentiation, 3T3-L1 cells were grown to confluence in DMEM (Mediatech) with 10% FBS, then in DM1 (DMEM with 10% FBS, 160 nM Insulin, 250 μM Dexamethasone, 0.5 mM Isobutylmethylxanthine) for 48 h, DM2 medium (DMEM with 10% FBS and 160 nM insulin) for another 48 h, and in DMEM containing 10% FBS for 2–6 more days, with medium change every 2 days. Intracellular lipids in adipocytes were visualized by staining with Oil Red O solution (60%).

Mouse mammary epithelial cell lines

HC-11 were grown in RPMI-1640 with 10% FBS, 5 μg/ml insulin, 10 ng/ml EGF, 2 g/l sodium bicarbonate, Penicillin–Streptomycin and 2.42 g/l HEPES; Comma 1D cells were grown in DMEM-F12 medium with 2% FBS, 10 μg/ml insulin, 5 ng/ml EGF, 0.3% BSA, Penicillin–Streptomycin and 2.42 g/l HEPES.

Preparation of conditioned media (CM)

Serum-free CM was collected from 3T3-L1 cells on days 10–14 of differentiation protocol overnight or after 48 h, (6 ml per 10 cm dish), and centrifuged at 2000 rpm for 10 min. When transfected 3T3-L1 cells were used, differentiated 3T3-L1 cells were detached from the culture dish and transfected with plasmid or siRNA solution using Amaxa Cell Line Nucleofector Kit L as described by the manufacturer, and inoculated into collagen I-coated plates. For vector or DN-RARα expression, 2 μg of vector or DN-RARα plasmid was added to 2×10^6 cells, and for siRNA transfection experiments, 2 μg of siRNA targeting pool was transfected into 2×10^6 cells using Amaxa nucleofector. siRNAs used were siCONTROL (Non-targeting siRNA pool, Cat. # D-001206, Dharmacon), PTHR siRNA (siGENOME SMART pool Cat. # M-042524, Dharmacon) and PTN siRNA (siGENOME SMART pool Cat. # M-042740, Dharmacon). Following 48 h of incubation of the transfected cells in growth medium, CM was collected as described earlier.