Molecular and Cellular Endocrinology 430 (2016) 1–11

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Retinoic acid induces nuclear FAK translocation and reduces breast cancer cell adhesion through Moesin, FAK, and Paxillin

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ARTICLE INFO

Article history:
Received 7 October 2015
Received in revised form
25 April 2016
Accepted 25 April 2016
Available online 26 April 2016

Keywords:
Retinoic acid
Moesin
FAK
Paxillin
Cell adhesion/migration
Breast cancer cells

ABSTRACT

Breast cancer is the most common malignancy in women, with metastases being the cause of death in 98%. In previous works we have demonstrated that retinoic acid (RA), the main retinoic acid receptor (RAR) ligand, is involved in the metastatic process by inhibiting migration through a reduced expression of the specific migration-related proteins Moesin, c-Src, and FAK. At present, our hypothesis is that RA also acts for short periods in a non-genomic action to cooperate with motility reduction and morphology of breast cancer cells. Here we identify that the administration of 10^{-6} M RA (10–20 min) induces the activation of the migration-related proteins Moesin, FAK, and Paxillin in T-47D breast cancer cells. The phosphorylation exerted by the selective agonists for RARα and RARβ, on Moesin, FAK, and Paxillin was comparable to the activation exerted by RA. The RARγ agonist only led to a weak activation, suggesting the involvement of RARα and RARβ in this pathway. We then treated the cells with different inhibitors that are involved in cell signaling to regulate the mechanisms of cell motility. RA failed to activate Moesin, FAK, and Paxillin in cells treated with Src inhibitor (PP2) and PI3K inhibitor (WM), suggesting the participation of Src-PI3K in this pathway. Treatment with 10^{-6} M RA for 20 min significantly decreased cell adhesion. However, when cells were treated with 10^{-6} M RA and FAK inhibitor, the RA did not significantly inhibit adhesion, suggesting a role of FAK in the adhesion inhibited by RA. By immunofluorescence and immunoblotting analysis we demonstrated that RA induced nuclear FAK translocation leading to a reduced cellular adhesion. These findings provide new information on the actions of RA for short periods. RA participates in cell adhesion and subsequent migration, modulating the relocation and activation of proteins involved in cell migration.

1. Introduction

Retinoic acid (RA), a pleiotropic signaling molecule derived from vitamin A, regulates critical genetic programs that control development, homeostasis, cell proliferation, and differentiation, as well as cell death or survival (Clagett-Dame and Knutson, 2011,Samarut and Rochette-Egly, 2012). This is the basis for the use of RA in cancer therapy (Altucci et al., 2007).

RA activity is primarily mediated by members of the retinoic acid receptor (RAR) subfamily, namely RARα, RARβ, and RARγ, which belong to the nuclear receptor superfamily. Classically, RARs function as ligand-inducible transcriptional regulators that heterodimerized with retinoid X receptors (RXRs). As such, they regulate the expression of subsets of target genes. Much of the research on the effects of retinoids is focused on the regulation of gene expression. Retinoids regulate the activity of a number of genes and proteins in many cell types, including growth factors, the extracellular matrix (ECM) proteins, and intracellular signaling molecules, such as protein kinase C and cyclic AMP-dependent protein kinases (Li et al., 2014,Scheibe et al., 1991, Sporn and Roberts, 1991,Zhang et al., 1996).

In addition to this scenario, recent studies highlighted a novel paradigm in which RA also induces the rapid activation of different signaling pathways (Al Tanoury et al., 2013). Studies from several laboratories have shown that RA rapidly and transiently activates several kinase cascades, which are exemplified by the mitogen

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http://dx.doi.org/10.1016/j.mce.2016.04.021
0303-7207/

activated kinase (MAPK) pathways. Indeed, RA activates p38 MAPK in fibroblasts, mouse embryo carcinoma cells, mammary breast tumor cells, and leukemia cells (Alsayed et al., 2001,Bruck et al., 2009,Gianni et al., 2002,Piskunov and Rochette-Egly, 2012). Most interestingly, the activation of p38 MAPK by RA occurs very rapidly (within minutes), suggesting a non-genomic action of RA and RARs, as described for steroid hormone receptors (Losel and Wehling, 2003).

The metastatic process requires the acquisition of invasive properties such as remodeling, adhesion, motility, and invasion of the ECM. Another important component of this process is the organization of cell adhesion sites that are directed by receptors, which physically link the ECM to the cytoplasmic actin cytoskeletal network and may transmit signals from the ECM to the cytoplasm.

The actin-binding protein Moesin, which belongs to the ezrin/radixin/Moesin (ERM) family, is a key regulator of ECM signals. Activated Moesin triggers the de-polymerization of actin fibers and the re-assembly of microfilaments toward the cell membrane edge, leading to the formation of cortical actin complexes and specialized cell membrane structures that are implicated in the generation of the cellular locomotive force (Louvet-Vallee, 2000).

The focal adhesion kinase (FAK) is another ECM controller. This non-receptor protein-tyrosine kinase is involved in cell attachment, migration, and invasion, which are crucial steps for cancer development and metastasis (Gabarra-Niecko et al., 2003,McLean et al., 2005). FAK is phosphorylated by c-Src, a non-receptor tyrosine kinase and then the recruitment of Src/FAK/PI3K complex occurred (Calalb et al., 1995). The activated Src/FAK/PI3K complex mediates the phosphorylation of multiple adhesion components involved in the dynamic regulation of cell motility. Considerable evidence suggests that enhanced protein tyrosine phosphorylation occurs during focal adhesion plaque formation. A specific tyrosine residue within another focal adhesion protein, Paxillin, has been identified as a primary target for phosphorylation by FAK (Parsons et al., 1994). FAK and Paxillin also show a high stoichiometry of tyrosine phosphorylation upon integrin activation (Bellis et al., 1995,Turner, 1994).

Retinoids have also been shown to regulate cell adhesion and migration. Some authors, including us, have demonstrated that RA and other biologically active retinoids inhibit cellular migration in several cell lines, such as human colon carcinoma cells (Woo and Jang, 2012) as well as human breast cancer MCF-7 and MDA-MB-231 cells (Dutta et al., 2009,Dutta et al., 2010). Our laboratory recently demonstrated that RA inhibits cellular migration by remodeling the actin cytoskeleton and down-regulating the expression of Moesin, Src, and FAK in T-47D and MCF7 breast cancer cells (Flamini et al., 2014).

At present, little is known about the possible mechanism by which retinoids affect breast cancer cell migration. We believe that retinoids act both non-genomically and genomically to regulate cellular processes. For this reason, in this work we aimed at deciphering the rapid non-genomic effects of RA on protein phosphorylation and actin cytoskeleton remodeling.

Recent studies revealed new aspects of FAK action in the nucleus where in a normal growth condition, the localization of FAK is prominent in focal adhesions and the cytosol. However, FAK mobilization occurred from integrin adhesion sites to the nucleus when cell de-adhesion occurred due to stress (Lim, 2013). In the nucleus, FAK interacts with tumor suppressor p53, resulting in p53 turnover to enhance cell survival (Lim, 2013).

We demonstrate that RA induces the activation of Moesin, FAK, and Paxillin through RARα and RARβ-selective retinoids in T-47D human breast cancer cells, which leads to nuclear FAK translocation and reduced cellular adhesion.

2. Materials and methods

2.1. Cell cultures and treatments

The human breast carcinoma cell line T-47D was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). T-47D cells were routinely grown in RPMI 1640 supplemented with L-glutamine (2 mM) and 10% fetal bovine serum. All-*trans*-retinoic acid (RA) was obtained from Sigma-Aldrich (St. Louis, MO). RA stock solution was dissolved in DMSO at a concentration of 10^{-2} M and maintained at −20 °C, protected from light, and in an inert atmosphere. We used a final concentration of 10^{-6} M of RA according to ours previous published results (Flamini et al., 2014). The synthetic agonist retinoids selective for RARα (BMS753), RARβ (BMS453), and RARγ (BMS961), and the synthetic antagonist retinoids selective for RARα (BMS195614) (Tocris Bioscience, USA) were kindly provided by Dr. Hinrich Gronemeyer (IGBMC, Illkirch, France). Agonist and antagonist retinoids were diluted in ethanol and added to the culture medium to give a final concentration of 10^{-6} M. The Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d) pyrimidine (PP2) was obtained from Calbiochem (EMD Biosciences, Germany); the PI3K inhibitor Wortmannin was from Sigma-Aldrich (Saint-Louis, MO); and the FAK inhibitor 14 (FAKi, CAS 4506-66-5, sc-203950A) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Whenever an inhibitor was used, the compound was added 30–45 min prior to starting the active treatments. The final concentration of the solvents was 1 μl of solvent per 1 ml of medium. In control cultures, the vehicle was added at the same final dilution. All experiments with retinoids were performed in reduced room light.

2.2. Immunoblotting

Cell lysates were separated by SDS-PAGE. In fractionation experiments, after treatments, the cells were washed in PBS and resuspended in lysis buffer for nuclear and cytoplasmic proteins extraction as reported by Andrews NC et al. (Andrews and Faller, 1991). The following antibodies were used: p-FAK Tyr^{397} and Moesin (BD Transduction Laboratories); Actin (C-11), p-FAK Tyr^{397} (sc-11765-R), p-Moesin Thr^{558} (sc-12895), Paxillin (sc-31010), p-Paxillin Tyr^{118} (sc-365020), RARα (sc-551), RARβ (sc-552) and p53 (sc-126) (Santa Cruz Biotechnology); HSP27 (also known as HSPB1), HSP72 (also known as HSPA1A) (Stressgen, USA); α-Tubulin (T9026) and HDAC1 (Sigma-Aldrich Laboratories). Primary and secondary antibodies were incubated with the membranes using standard techniques. Inmunodetection was accomplished using enhanced chemiluminescence. The images were captured using ChemiDoc™ XRS + System with Image Lab™ Software #170-8265 (Biorad, USA).

2.3. Transfection experiments

The synthetic small interfering RNA for RARβ (sc-29466) was from Santa Cruz Biotechnology. T-47D cells (60–70% confluent) were transfected with 50–75 nM of target siRNA using Lipofectamine (Invitrogen- Thermo Fisher Scientific, USA). The cells were treated 24 h after siRNA transfection. Transfection efficiency was checked for expression of RARβ by immunoblotting.

2.4. Plasmid construction

To obtain the RARα silencing construct, a custom-synthesized double-stranded DNA coding for a RARα-targeting shRNA (5′-GATCCGCGGGCACCTCAATGGGTACTTCCTGTCAGA-TACCCATTGAGGTGCCCGCTTTTTG-3', the underlined sequence