Retinoic Acid Increases Tyrosine Phosphorylation of Focal Adhesion Kinase and Paxillin in MCF-7 Human Breast Cancer Cells

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ABSTRACT

Treatment of estrogen receptor (ER)-positive MCF-7 human breast cancer cells with retinoic acid (RA) inhibited cell growth and increased cell adhesion to fibronectin. In contrast, ER− MDA-MB-231 cells failed to respond. Western blot analysis showed that tyrosine phosphorylation of two major bands at Mr 125,000 and Mr 68,000 was induced by RA in ER+ MCF-7 human breast carcinoma cells. However, this induction was a late phenomenon detectable at 12 and 24 h, but not within 3 h. A similar increase of tyrosine phosphorylation by RA was observed in ER+ human breast cancer cell lines T-47D and ZR-75-1, but not in the ER− cell lines MDA-MB-231, MDA-MB-453, and MDA-MB-468. Focal adhesion kinase and paxillin, which localize in focal adhesion plaques and may play important roles in the integrin signaling pathway, were identified as the major proteins showing RA-induced tyrosine phosphorylation. The retinoid X receptor-selective compound SR11237 failed to induce tyrosine phosphorylation, indicating that retinoid X receptor activation is not involved in this phenomenon. In contrast, stable overexpression of a truncated RA receptor (RAR) α cDNA, RARα0403, with strong RAR dominant negative activity prevented the increase in tyrosine phosphate, suggesting that RAR signaling is involved in RA-induced tyrosine phosphorylation. Tyrosine phosphorylation was induced the most by the RAR-α (193836), followed by RAR-γ (194433), but was not significantly induced by RAR-β (193174)-selective retinoids. This study demonstrates a coordinated albeit relatively late effect of RA on cell adhesion and tyrosine phosphorylation in ER+ human breast cancer cells and suggests RAR-α as the major responsible retinoid receptor.

INTRODUCTION

Retinoids, the natural and synthetic vitamin A derivatives, have been extensively shown to modulate growth, differentiation, and development (1, 2). Their effects are generally mediated by two classes of nuclear receptors, the RARs and RXRs, encoded by six related genes, each of which generates distinct proteins (3, 4). Much of the research on the effects of retinoids is focused on the regulation of gene expression. Retinoids regulate the activities of a number of genes and proteins in many cell types, including growth factors, ECM proteins, and intracellular signaling molecules such as protein kinase C and cyclic AMP-dependent protein kinases (5–11). Recent data showed that RA can induce the phosphorylation of the E1A-associated Mr 300,000 protein during the differentiation of F9 embryonal carcinoma cells (12).

Retinoids have also been shown to regulate cell adhesion (13), which plays a central role in diverse cellular events, including cellular differentiation, development, and the process of carcinogenesis. The organization of cell adhesion sites is directed by a family of transmembrane receptors known as integrins, which are heterodimeric transmembrane receptors comprised of α and β subunits, which physically link the ECM to the cytoplasmic actin cytoskeletal network and may function to transmit signals from the ECM to the cytoplasm (14). FAK, a cytosolic tyrosine kinase localized in focal adhesion plaques, plays a central role in integrin-mediated signal transduction (15). Considerable evidence suggests that enhanced protein tyrosine phosphorylation occurs during focal adhesion plaque formation, and that this phenomenon is mediated through transmembrane integrin molecules. A specific tyrosine residue within another focal adhesion protein, paxillin, has been identified as a primary target for phosphorylation by FAK (16). FAK and paxillin also demonstrate a high stoichiometry of tyrosine phosphorylation upon integrin activation (17, 18). Additionally, the increased tyrosine phosphorylation of FAK and/or paxillin have been found to be responsive to a variety of stimuli, including lysophosphatidic acid (19), angiotensin II (20), epidermal growth factor (21), platelet-derived growth factor (22), hepatocyte growth factor (23), nerve growth factor (24), and the neuropeptides bombesin, endothelin, and vasopressin (25, 26).

Our previous results showed that RA can increase integrin β1 synthesis in F9 teratocarcinoma cells (13) and down-regulate the expression of fibronectin in NIH-3T3 cells (8). This work led us to identify the possible regulatory effects of RA on integrin signaling. In this study, we have demonstrated RA induction of tyrosine phosphorylation and then identified FAK and paxillin as the major proteins showing RA-induced tyrosine phosphorylation in ER+ but not ER− human breast carcinoma cells. We also show that RAR-α and RAR-γ-selective retinoids are active in this pathway in MCF-7 human breast cancer cells.

MATERIALS AND METHODS

Materials. DMEM and FBS were obtained from Biofluid. All-trans-RA was purchased from Sigma. Monoclonal anti-phosphotyrosine antibody 4G10 and polyclonal anti-FAK antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-FAK antibody and anti-paxillin antibody were purchased from Transduction Laboratories (Lexington, KY). Protein A-agarose was from Boehringer Mannheim (Mannheim, Germany). Prestained protein molecular weight standards (range, Mr 14,300–200,000) were obtained from Life Technologies, Inc. The horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescent Western blotting system were obtained from Amersham. Other reagents and chemicals were from Sigma.

Cell Lines and Cell Culture. ER+ human breast cancer cell lines MCF-7, ZR-75-1, and T-47D and ER− cell lines MDA-MB-231, MDA-MB-453, and MDA-MB-468 were obtained from the American Type Culture Collection (Rockville, MD) and routinely maintained in DMEM supplemented with 10% FBS in a humidified atmosphere containing 5% CO2 at 37°C.

Retinoid Treatment. Human breast cancer cells were seeded onto cell culture dishes and kept in DMEM with 10% FBS for 24 h. The medium was changed to 0.5% FCS-containing DMEM, and the cells were incubated for an additional 24 h and then treated with 1 μM (or as specified) all-trans-RA (Sigma) or RAR-selective retinoids (RXR-selective SR11237 was obtained

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4 The abbreviations used are: RAR, retinoic acid receptor; ER, estrogen receptor; RA, retinoic acid; FAK, focal adhesion kinase; RXR, retinoid X receptor; ECM, extracellular matrix; FBS, fetal bovine serum; RIPA, radioimmunoprecipitation assay; CRBP, cellular retinol-binding protein.
from Dr. Keiko Ozato, NIH, Bethesda, MD) as indicated or with DMSO for the indicated time.

**Cell Attachment Assay.** Measurements of cell attachment were done as described previously (27). Cells (5 x 10^3) were grown in 10-cm (diameter) dishes in DMEM with 10% FBS for 24 h. The medium was then changed to 0.5% FCS-containing DMEM, and the cells were grown in this medium for an additional 16 h before treatment with 1 x 10^{-6} M RA or the solvent DMSO for different times up to 72 h. Cells (2.5 x 10^5) in 100 μl of medium were added to each well of 96-well microwell culture clusters precoated with fibronectin (Dickson) and incubated at 37°C for 1, 2, 3, 4, and 5 h. After removing unattached cells, each well was rinsed with PBS, and 10 μg of fluorescent substrate 4-methyl-umbelliferylheptanoate (Sigma)/100 μl were added. The cell culture plates were incubated at 37°C for 30 min, followed by measurement by using a Dynatech Instruments Microfluor Reader.

**Western Blotting.** RA- or DMSO-treated cells were washed and lysed in Laemmli buffer without reducing agent and bromphenol blue. Whole cell lysates were boiled for 5 min and centrifuged to remove insoluble cell debris. Protein concentration was determined by the bicinchoninic acid method (Pierce). β-Mercaptoethanol and saturated solution bromphenol blue were added to the samples at a 1% final concentration. Equal amounts of protein were then loaded onto 4–15% polyacrylamide gels. The proteins were transferred to supported nitrocellulose membrane on a Bio-Rad electroblot apparatus. For the detection of phosphotyrosine, the blots were incubated in 3% nonfat milk in PBS at room temperature for 1 h with constant agitation. The anti-Tyr(P) antibody was used at 1 μg/ml in 2% milk, and the blots were incubated at 4°C overnight. For the detection of other proteins, the blots were blocked at 4°C using 5% nonfat milk overnight in TBS [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and sodium fluoride, and 1 μg/ml aprotinin, leupeptin, and pepstatin]. Lysates were clarified by centrifugation, the supernatants were transferred to tubes for immunoprecipitation. Immunoprecipitation was routinely performed by adding 4 μg of polyclonal anti-FAK or monoclonal anti-paxillin antibody to 250 μg of lysates (in 500 μl of RIPA buffer). The reaction mixture was incubated at 4°C for 1 h, and 50 μl of protein A-agarose beads were added to the mixture, followed by incubation at 4°C overnight on a rotary shaker. The immunoprecipitates were washed three times with ice-cold RIPA buffer and collected by a microcentrifuge pulse, and the proteins were extracted with 50 μl of 2× Laemmli sample buffer. The samples were electrophoretically separated on SDS-polyacrylamide 4–15% gradient gels and immunoblotted with anti-FAK, paxillin, or Tyr(P) antibody.

**Infection of MCF-7 Cells with Retroviral Vectors.** The retroviral vector LXRARα403SN, in which a truncated RAR-α gene is inserted into the retroviral vector LXSN, was a gift from Dr. Steven J. Collins (28, 29). Cells were seeded at 50% confluence into 100-mm dishes. The next day, they were infected with the LXSN or LXRARαSN retroviral vector in the presence of 4 μg/ml Polybrene (8). After an overnight incubation, the medium was replaced, and cells were grown for 48 h before G418 (1 mg/ml) was added. G418-resistant cells were isolated.

Northern blot analysis was used to identify LXRARαSN- or LXSN-transfected cell clones. The full-length fragments of the RAR-α were excised from the expression plasmid pSG5-RAR-α. Isolation of total RNA was performed by using TRizol LS reagent from Life Technologies, Inc. Total RNA (20 μg) was fractionated on a 1% agarose gel and blotted overnight onto Schleicher & Schuell nitrocellulose. The membrane was prehybridized for 5 h at 42°C in a prehybridization solution of 6× SSC, 5× Denhardt’s reagent, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA, and 50% formamide. The probes (5 x 10^6 cpm/ml) were boiled and added to the prehybridization buffer, and the membranes were hybridized for 24 h at 42°C. After washing, autoradiography was performed on Kodak X-Omat AR film using double exposing screens.

**RESULTS**

**RA Increases Adhesion of ER+ Human Breast Cancer MCF-7 Cells.** Fig. 1A shows that RA treatment of MCF-7 cells under the conditions described in “Materials and Methods” resulted in an increased percentage of cells attached to fibronectin. This increased attachment could be observed at 72 h of RA treatment. The attachment to fibronectin of the ER− cell line MDA-MB-231 was unaffected (Fig. 1B).
RA Induces Tyrosine Phosphorylation in ER\(^1\) but not ER\(^2\) Human Breast Cancer Cell Lines.

MCF-7 cells were treated with RA or DMSO, followed by immunoblotting of whole cell lysates with an anti-phosphotyrosine antibody. RA induced tyrosine phosphorylation of two major bands (Fig. 2A) at around \(M_r 68,000\) (band 2, showing a 3-fold and a 3.7-fold induction at 12 and 24 h, respectively, Fig. 2B) and a doublet at about \(M_r 125,000\) (band 1, showing a 5.6-fold and an 8.5-fold induction at 12 and 24 h, respectively, Fig. 2B). The effect of RA was visible at \(10^{-8}\) M, but \(10^{-7}\) M or higher concentrations were most active (Fig. 2C). Similar albeit lesser effects of RA on tyrosine phosphorylation could also be observed in two other ER\(^+\) human breast cancer cells but fails to significantly increase tyrosine phosphorylation in ER\(^-\) MDA-MB-468, MDA-MB-231, and MDA-MB-453 breast cancer cells. 

Fig. 2. A, RA increases tyrosine phosphorylation in MCF-7 cells. Total cell lysates were obtained from MCF-7 cells treated with RA (1 m M) or vehicle (DMSO). Protein (20 \(\mu\)g) from cell lysates was electrophoretically separated on a polyacrylamide 4–15% gradient gel and immunoblotted with monoclonal anti-phosphotyrosine antibody. This experiment was repeated three times with similar results. B, densitometric analysis of Western blot. C, dependence of tyrosine phosphorylation on RA concentration. D, RA increases tyrosine phosphorylation in ER\(^+\) ZR-75-1 and T-47D human breast cancer cells but fails to significantly increase tyrosine phosphorylation in ER\(^-\) MDA-MB-468, MDA-MB-231, and MDA-MB-453 breast cancer cells. E, densitometric analysis of the data shown in D.

RA Induces Tyrosine Phosphorylation in ER\(^+\) but not ER\(^-\) Human Breast Cancer Cell Lines. MCF-7 cells were treated with RA or DMSO, followed by immunoblotting of whole cell lysates with an anti-phosphotyrosine antibody. RA induced tyrosine phosphorylation of two major bands (Fig. 2A) at around \(M_r 68,000\) (band 2, showing a 3-fold and a 3.7-fold induction at 12 and 24 h, respectively, Fig. 2B) and a doublet at about \(M_r 125,000\) (band 1, showing a 5.6-fold and an 8.5-fold induction at 12 and 24 h, respectively, Fig. 2B). The effect of RA was visible at \(10^{-8}\) M, but \(10^{-7}\) M or higher concentrations were most active (Fig. 2C). Similar albeit lesser effects of RA on tyrosine phosphorylation could also be observed in two other ER\(^+\) human breast cancer cell lines (ZR-75-1 with a 1.7-fold and a 2.2-fold increase for bands 1 and 2, Fig. 2D) and T-47D with a 2-fold and a 2.2-fold increase for bands 1 and 2, respectively; and T-47D with a 2-fold and a 2.2-fold increase for bands 1 and 2,
Role of RAR Signal Transduction Pathways on RA-induced Tyrosine Phosphorylation in MCF-7 Cells. To explore the possible mechanism involved in RA-induced tyrosine phosphorylation in MCF-7 cells, SR11237, a RXR-selective compound, was used to examine RXR involvement. SR11237 specifically activates reporter genes fused to the RXR-responsive element of the CRBPII promoter to which RXR-RXR homodimers bind and is unable to induce genes driven by a RAR-responsive element, such as the RA response element of RAR-β and CRBPI (30). SR11237 could not induce tyrosine phosphorylation of MCF-7 cells (data not shown), indicating that RXR is not involved in RA-induced tyrosine phosphorylation.

An additional approach was to use MCF-7 cells expressing the RARα403 construct transduced by a retroviral vector. Fig. 3 shows that the infected cells express the typical 4.7-kb retroviral transcript containing the RARα403 mRNA. This construct functions as a dominant negative against RAR-mediated responses (28, 29). RA did not increase tyrosine phosphorylation in the dominant negative RARα403-transfected MCF-7 cells (Fig. 4A), whereas RA could still increase tyrosine phosphorylation in the control LXSN vector-transfected MCF-7 cells (Fig. 4B). These results indicate that tyrosine phosphorylation in MCF-7 cells is mediated by RAR.

FAK and Paxillin Are the Major Proteins Showing RA-induced Tyrosine Phosphorylation. We were interested in identifying the major proteins showing RA-induced tyrosine phosphorylation. For this purpose, we chose several proteins with M, around 125,000 and 68,000 as the candidates. Anti-c-Src (M, 60,000), Fyn (M, 59,000), and Lyn (M, 56,000) antibodies were chosen to immunoprecipitate the proteins with M, around 68,000 and detect their tyrosine phosphorylation. Anti-JAK1 (M, 130,000), retinoblastoma protein (pRB; M, 110,000), and vinculin (M, 116,000) were chosen to immunoprecipitate the proteins with M, around 125,000 and detect their tyrosine phosphorylation. No obvious increase in tyrosine phosphorylation of those proteins at the indicated time points was found after RA treatment (data not shown). Immunoprecipitation of FAK and paxillin was followed by immunoblotting with the respective antibody used for immunoprecipitation to detect the expression of FAK and paxillin. The results from Fig. 5, A and B, show that RA has no obvious effect on FAK and paxillin expression at 12 and 24 h. In sharp contrast, tyrosine phosphorylation of FAK (Fig. 5B) and paxillin (Fig. 5D) increased by 2.8- and 2.9-fold at 12 and 24 h, respectively, for FAK (Fig. 6B) and by 2.1- and 4-fold for paxillin (Fig. 6D). These experiments were conducted on cell lysates that were immunoprecipitated with anti-FAK or paxillin antibody, followed by immunoblotting with anti-Tyr(P) antibody. The results presented above demonstrate that FAK and paxillin are major proteins showing a RA-induced tyrosine phosphorylation in MCF-7 cells. However, the phosphorylation of other proteins is likely, especially because only a single band is observed in Fig. 5, whereas a doublet was observed in Fig. 2.

Fig. 4. A, RA-induced tyrosine phosphorylation in MCF-7 cells is mediated by RARs. Total cell lysates were obtained from LXRARα403SN (A)- or LXSN (B)-transfected MCF-7 cells. Protein (20 µg) from each sample was analyzed in SDS-PAGE 4–15% gradient gel, and Western blots were probed with anti-phosphotyrosine antibody. C, RAR-α-selective and RAR-γ-selective retinoids, but not RAR-β-selective retinoids, stimulate tyrosine phosphorylation. The RAR-selective retinoids 193836 (RAR-α selective), 194433 (RAR-γ selective), and 193174 (RAR-β selective) were tested under the same conditions as described for RA at a 1 µM concentration. Densitometry was performed as indicated above. The experiment was done in triplicate, and representative results are shown. Variance between samples was less than 15% in densitometry readings.
Interestingly, insulin was found to stimulate tyrosine dephosphorylation of FAK (37). The induction of FAK and paxillin tyrosine phosphorylation by the factors mentioned above is rapid. In contrast, RA did not show tyrosine phosphorylation induction for at least 3 h. Induction, however, was reproducibly detected at 12 and 24 h. The reason for this relatively late response and the mechanism by which RA induces FAK and paxillin tyrosine phosphorylation have yet to be determined. Many of the actions of retinoids are mediated through two classes of nuclear receptors, RARs and RXRs, as well as by cytoplasmic binding proteins (cellular retinoic acid-binding proteins and CRBPs). The RARs and RXRs act as ligand-inducible transcription factors that can increase the transcription of direct target genes by binding to cis-acting RA response elements on DNA (38). Several studies demonstrated that ER+ human breast carcinoma cell lines and tumor samples exhibit significantly higher levels of RAR-α than their ER− counterparts. ER+ human breast carcinoma cell lines are sensitive, and ER− cell lines are resistant to growth-inhibitory effects of RA (39, 40). Retinoid-resistant ER− human breast carcinoma MDA-MB-231 cells transfected with RAR-α acquired sensitivity to growth inhibition by retinoids (41). These results suggested that RAR-α may have great importance in mediating RA growth-inhibitory effects in breast cancer cells. In this work, RA-mediated repression of the RA-induced tyrosine phosphorylation was also demonstrated. The introduction of a mutated RAR-α (RARE403), which has strong dominant-negative activity on the RAR-mediated signaling pathways, abolished the RA induction of tyrosine phosphorylation in MCF-7 cells. Furthermore, the RXR-selective compound, SR11237, was unable to induce tyrosine phosphorylation in MCF-7 cells. The expression of the RAR-α gene was not sufficient to reestablish RA-induced tyrosine phosphorylation in RA-refractory ER− MDA-MB-231 cells (data not shown). Our data show that RAR-α and RAR-γ-selective retinoids are at least as active as RA in eliciting the tyrosine-phosphorylation response; however, RAR-β does not appear to be involved.

The delayed response suggests that RA may first modulate the expressions of its direct target genes, such as growth factors or other signal transducers, and induce the tyrosine phosphorylation of FAK and paxillin as a result of these or related primary events. However, under similar culture conditions, RA inhibits the cell growth of ER+ but not ER− breast cancer cells. The cell growth inhibition is accom-
panied by a marked down-regulation of cyclin D3 (42) expression (19, 62, and 83% inhibition at 24, 48, and 72 h, respectively), simultaneous with the reduced expression of the retinoblastoma protein pRB and its phosphorylated form and of CDK4 and E2F1. Significant inhibition of cell growth is observed at 72 h of RA treatment, but not at 24 and 48 h of RA treatment. Therefore, the marked stimulation of FAK and paxillin tyrosine phosphorylation appears to precede the observed down-regulation of cyclin D3, CDK4, pRB as well as E2F1 proteins and the observed inhibition of cell growth and increase in cell adhesion that could not be detected before 72 h. Therefore, we conclude that RA-induced tyrosine phosphorylation may be a responsible factor in the cascade that leads to the inhibition of cell growth and increased adhesion and apoptosis.

Finally, it is also of interest that a very recent report (43) has shown that RA augments mitogen-activated protein/extracellular signal-regulated kinase-dependent mitogen-activated protein kinase activation needed to elicit HL-60 cell differentiation and growth arrest. Obviously, several phosphorylation pathways signal downstream of the retinoid receptors.

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