HOXA5 Acts Directly Downstream of Retinoic Acid Receptor β and Contributes to Retinoic Acid–Induced Apoptosis and Growth Inhibition

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Abstract

The promise of retinoids as chemopreventive agents in breast cancer is based on the differentiation and apoptosis induced upon their binding to the retinoic acid (RA) receptor β (RARβ). We have previously shown that HOXA5 induces apoptosis in breast cancer cells. In this study, we investigated whether RA-RARβ and HOXA5 actions intercede to induce apoptosis and differentiation in breast cancer cells. We found that HOXA5 expression can be induced by RA only in RARβ-positive breast cancer cells. We have, for the first time, identified the RA response element in HOXA5, which was found to be located in the 3′ end of the gene. Chromatin immunoprecipitation assays showed that RARβ binds directly to this region in vivo. Overexpression of RARβ strongly enhances RA responsiveness, and knocking down RARβ expression abolishes RA-mediated induction of HOXA5 expression in breast cancer cells. In addition, there is coordinated loss of both HOXA5 and RARβ expression during neoplastic transformation and progression in the breast epithelial cell model, MCF10A. Knockdown of HOXA5 expression partially abrogates retinoid-induced apoptosis and promotes cell survival upon RA treatment. These results strongly suggest that HOXA5 acts directly downstream of RARβ and may contribute to retinoid-induced anticancer and chemopreventive effects.

Introduction

Retinoids, the natural and synthetic derivatives of vitamin A, exert profound effects on cell growth, differentiation, apoptosis, and morphogenesis (1). They are currently used in the treatment of epithelial cancer and promyelocytic leukemia and are being evaluated as preventive and therapeutic agents for a variety of human cancers, including breast cancer (2–4). The effects of retinoids are mainly mediated by two classes of nuclear receptors, the retinoic acid (RA) receptors (RAR) and retinoid X receptors (RXR; refs. 1, 5). 9−cis-RA is a high-affinity natural ligand for both RARs and RXRs (6), whereas all-trans-RA is a high-affinity natural ligand only for the RARs (7). RARs and RXRs are each encoded by three distinct genes (α, β, γ), which function as ligand-activated transcription factors (1). RARs interact with RXRs, forming RXR-RAR heterodimers that bind to RA response elements (RARE) to control the expression of RA-responsive genes in the presence of retinoids (1). Some of the target genes are RARs themselves, in particular the RARβ gene, for which a RARE (βRARE) was identified in the promoter region (8).

Altered nuclear receptor activities are associated with carcinogenesis. In particular, loss of RARβ expression was found in a number of malignancies, including carcinomas of lung and breast, and squamous cell carcinomas of the head and neck (9–11). A growing body of literature has shown that the anticancer effect of RA is primarily mediated by RARβ, which is a potent tumor-suppressor protein (12, 13). For example, inhibition of RARβ expression in RARβ-positive cancer cells abolished the RA-induced apoptotic effect (14). Conversely, reexpression of RARβ in RARβ-negative cancer cells restored RA-induced growth inhibition, apoptosis, and decreased tumorigenicity (12, 15). In phase I/II clinical trials of breast cancer testing a combination of all-trans-RA and tamoxifen, RARβ expression was found to be consistently elevated in the breast tissue (16). However, how RARβ exerts its anticancer activity is still largely unknown.

HOX gene expression is regulated by RA in embryonic cells and tissues (17). In vertebrates, there are 39 HOX genes that are organized into four clusters (i.e., HOXA, HOXB, HOXC, and HOXD; ref. 18). In embryonic carcinoma cells, expression of the entire HOXB gene cluster can be induced by RA treatment (19). In contrast, in differentiated cells and tumor cells, very few HOX genes can be induced by RA (20). Despite efforts to identify the RAREs for HOX genes, only five RAREs have been identified for 5 of 39 HOX genes (HOXA1, HOXB1, HOXA4, HOXB4, and HOXD4; refs. 21–24). HOXA5 expression has been shown to be regulated by RA in embryonic carcinoma cells and in developing mouse lung (25, 26). No RARE has yet been identified for HOXA5. How HOXA5 expression is regulated by RA and the role of HOX gene in RA-mediated cellular function is poorly understood.

Recently, our laboratory has shown that HOXA5 expression is lost in 60% breast cancer cell lines and primary tumors (27, 28). Overexpression of HOXA5 in breast cancer cells induced apoptosis (27, 29). To determine if HOXA5 expression can be reactivated by RA, we treated a variety of breast cancer cells with RA and its analogues. We found that HOXA5 expression can be induced only in RARβ-positive breast cancer cells. Further, we identified the RARE to which RARβ binds directly. We also showed that induction of HOXA5 is important for RA-mediated apoptosis and cellular growth inhibition. This study suggested that HOXA5 acts directly downstream of RARβ and plays an important role in RA-mediated anticancer activity.

Materials and Methods

Reagents and cell culture. All of the retinoids (all-trans-RA, 9-cis-RA, 13-cis-RA, and 4-HPR) were purchased from Sigma-Aldrich. RARβ

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antagonist LE135 was kindly provided by Dr. Hiroyuki Kagechika (Tokyo Medical and Dental University, Tokyo, Japan). Western blot analysis was done using standard procedure with antibodies to HOX5 (Zymed) and RARβ (Santa Cruz Biotechnology).

All breast cancer cell lines except MCF10A were maintained in DMEM with 10% fetal bovine serum. MCF10A were maintained in DMEM/F12 containing 5% horse serum, 0.1% non-essential amino acids, 100 μg/mL streptomycin, and 100 μg/mL chlora toxin. MCF10A, T1K.C12, and MCFCA.1h were obtained from Fred Miller and Robert Pupey (Karmanos Cancer Institute, Detroit, MI) and were cultured in MCF10A medium (30).

Plasmids and transfection process. The pLe-P1-Luc was constructed by replacing the cytomegalovirus promoter sequence of the reporter plasmid pLe-Luc with HOX5 promoter sequence, which was amplified using PCR with forward primer 5'-GAGATCTAACCTCCTTGCTTGGC-3' and reverse primer 5'-CAAGCTTTGTGGCTCGGCTTTG-3'. The plc-A5GF3-Luc was generated by inserting 6.5 kb of HOX5 downstream sequence into pLe-P1-Luc vector. To construct the deletion plasmid D1, plc-A5GF3-Luc was digested with (Ndel) and (EcoRI) to release a small fragment of the downstream sequence following by filling with Klenow enzyme and self-ligation with T4 ligase. Similarly, plc-A5GF3-Luc was cut with EcoRI/Ndel to construct D2, BsaBI/Ndel to construct D3, KpnI/Al III to construct D4, and KpnI/MluI to construct D5. The deletion sequences in F1 to F4 was PCR amplified with primers (F1 forward primer 5'-AGGTACCATGAGAACACTCCAGCTCAG-3'; F2 forward primer 5'-AGGTACCATGAGGGTACAGATTGGG-3'; F3 forward primer 5'-AGGTACCATGCTGCATCCTGGGTACAA-3'; F4 forward primer 5'-AGGTACCGTGCTAATACAGAAGGGTGCT-3' and reverse primer 5'-CATACATGAACACATGAGGTCACC-CTGGT-3') and cloned into D1 at the KpnI/MluI sites. The oligonucleotides containing one or three copies of either site I or II were synthesized and inserted into pG3-Promoter vector to generate I-1, I-3, II-1, and II-3 plasmids. Similarly, II-3M, which contained three copies of mutated site II sequence, was constructed. The same mutations were introduced into F1 construct to generate M1 and M2 constructs, respectively.

Transient transfection experiments were done as described previously (31). At 24 h posttransfection, the cells were treated with 1 μmol/L of RA for another 24 h before harvesting for luciferase assay according to the manufacturer's instructions (Promega).

Establishment of HOX5-short hairpin RNA stably transfected cell lines. The HOX5 short hairpin RNA (shRNA)-expressing plasmid was constructed by inserting the synthesized oligonucleotides (oligo 1a: 5'-GATCTCCAGGTCGCGCCCTA-3' and oligo 1b: 5'-AGATCAAGGGCGCAGGAGATC-3'), which are stably expressed in breast cells (33, 34), we examined the expression status during mammary carcinogenesis and progression, and RXRβ is not detected in breast cells (33, 34), we examined the expression status of RARs in normal breast and breast cancer cells, RA to HOX5 signaling pathway was specifically blocked in most breast cancer cells, we tested RA response using reporter plasmid containing RARE in the promoter. The results indicated that most of the breast cancer cell lines, except for MDAMB231 cells, responded to RA treatment (Supplementary Fig. S1). A few cell lines, such as 293T cells, were expressed in almost all of the 12 breast cancer cell lines examined, although the expression level varied from cell line to cell line. However, RARβ expression was detected in only four cell lines (MCF10A, MCF7, MDAMB435, and HS578T cells; Fig. 1B). Interestingly, HOX5 expression was induced by RA in three of these four RARβ-positive cell lines, suggesting that RARβ expression is important for HOX5 induction.

Consistent with this finding, we found that all-trans-RA, which specifically binds to RARs, strongly induced HOX5 expression.

Results

Induction of HOX5 expression by RA in breast cancer cells. With a goal to reactivate the expression of HOX5 and inhibit breast cancer cell growth, we treated a variety of breast cancer cells with different concentrations of RA. We found that HOX5 expression was induced in three of eight breast cancer cell lines tested (Fig. 1A). To gain additional evidence to support the concept that RA/HOXA5 signaling pathway was specifically blocked in most breast cancer cells, we tested RA response using reporter plasmid containing RARE in the promoter. The results indicated that most of the breast cancer cell lines, except for MDAMB231 cells, responded to RA treatment (Supplementary Fig. S1). A few cell lines, such as 293T cells, were expressed in almost all of the breast cancer cell lines examined, although the expression level varied from cell line to cell line. However, RARβ expression was detected in only four cell lines (MCF10A, MCF7, MDAMB435, and HS578T cells; Fig. 1B). Interestingly, HOX5 expression was induced by RA in three of these four RARβ-positive cell lines, suggesting that RARβ expression is important for HOX5 induction.

Consistent with this finding, we found that all-trans-RA, which specifically binds to RARs, strongly induced HOX5 expression.
of HOXA5 coding region, we added the 6.5 kb 3'-end DNA sequence to our construct (pLC-A5GF3-Luc; Fig. 2A). The new construct showed a strong response to RA treatment, indicating that the RARE is located in this 3'-end fragment (Fig. 2B).

To identify the RARE in this 6.5-kb fragment, we generated a series of deletion constructs. Transient transfection assays clearly showed that the ability of the reporter constructs to respond to RA depended on a 300-bp fragment located between the 5' ends of D5 and F4 constructs (Fig. 2C). The sequence of this 300-bp fragment revealed two potential RAREs, which match or closely resemble the RA half-site consensus sequence AGGTCA. To analyze the role of these two potential binding sites in RA responsiveness, point mutations were introduced into each of these two sites. Mutation of site I had no dramatic effects on the responsiveness to RA, whereas the mutation of site II significantly blocked the increase in reporter activity in response to RA treatment (Fig. 2C). To confirm that the site II sequence is a functional RARE, one or three copies of the 13-bp sites I and 15-bp site II nucleotides were synthesized, and each was inserted in front of a SV40 promoter (Fig. 2D). Transient transfection assay showed that the second binding site, but not the first, conferred responsiveness to RA. Further, mutation of this putative binding motif completely abolished the responsiveness to RA treatment (Fig. 2D).

RARβ directly binds to the RARE and mediates HOXA5 induction in breast cancer cells. To further confirm that RARβ expression is important for HOXA5 induction, we cotransfected the reporter construct (pLC-A5GF3-luc) with RAR- and RXRβ-expressing vectors into CV-1 cells. CV-1 monkey kidney fibroblast cells are widely used for testing RAR isotype-specific activities, partly due to the low or absent endogenous level of expression of all of the RARs (7, 39, 40). Although expression of each of the RARs and RXRα restored HOXA5 RARE function to varying levels in CV-1 cells, RARβ expression most strongly up-regulated the response of HOXA5 RARE to RA treatment (Fig. 3A). Conversely, knocking down of RARβ expression in breast cancer cell line Hs578T cells using siRNA blunted the induction of HOXA5 expression by RA (Fig. 3B). Further, ChIP assays confirmed that both RARα and RARβ bound strongly to the site II–containing DNA region in vivo, whereas RARγ bound weakly to this region (Fig. 3C). These experiments provide strong support for the hypothesis that RARβ expression is required for HOXA5 induction in breast cancer cells.

Because the expression of both RARβ and HOXA5 has been reported to be lost during breast cancer progression, we wanted to test whether there is a correlation between the expression patterns of these two genes. We examined the expression status of HOXA5 and RARβ in a MCF10A model of breast cancer development and progression using real-time PCR (Fig. 3D). Each of these cell lines is derived from parental MCF10A, an immortalized normal breast epithelial cell line. Upon transplantation into immunodeficient mice, MCF10A cells do not grow; T1k.C12 grow slowly and develop morphologic structures similar to the hyperplastic lesions and carcinoma in situ of the human breast; whereas MCFCA.1h cells are malignant and develop tumors that infiltrate the surrounding tissues, thus recapitulating multiple steps of progression in breast cancer (30). MCF10A cells express both RARβ and HOXA5. During tumor progression in this model, the expression of both HOXA5 and RARβ were coordinately lost, suggesting a correlation between the loss of HOXA5 and RARβ in a biologically relevant model system (Fig. 3D). Due to the relatively low-level expression of RARβ in each member of the MCF10A series of cells, the reduction in RARβ expression at the mRNA level does not seem to be so dramatic. However, previous studies, using the same model system, have shown that progression and malignant transformation of MCF10A cells is associated with loss of the induction of RARβ at both transcription and translation levels by RA (41).

Figure 1. RA-induced HOXA5 expression in breast cancer cells. A, Western blot analysis of eight breast cancer cell lines treated with different concentrations of all-trans-RA (0, 0.01, 0.1, and 1 μm/L) for 24 h. B, examination of expression of the three RARs in breast cancer cell lines using RT-PCR. C, induction of HOXA5 expression in MCF7 cells with RA alone or in combination with LE135 for 24 h before harvesting for Western blot analysis. *, nonspecific band.
HOXA5 induction contributes to RA-induced apoptosis and cellular growth inhibition. RARβ has been shown to mediate the growth-inhibitory effect of RA by promoting apoptosis in breast cancer cells (12, 15). In this study, we have presented evidence that RARβ directly regulated HOXA5 expression in breast cancer cells (Fig. 2D). To test whether HOXA5 induction is functionally important for RA-mediated cellular growth inhibition, we generated a MCF7 breast cancer cell line stably transfected with HOAX5 shRNA. In scrambled shRNA-transfected cells, HOXA5 expression was strongly induced by RA. In contrast, in HOXA5-specific shRNA transfected cells, induction of HOXA5 expression was greatly decreased (Fig. 4A). Blockage of HOXA5 induction also partially inhibited RA-induced apoptosis. Treatment for 6 days with 1 μmol/L of RA resulted in apoptosis of 8.1% of scrambled shRNA-transfected cells and 3.8% of HOAX5 shRNA-transfected cells (Fig. 4B). Consistent with this finding, clonogenic assays showed that HOAX5 shRNA-transfected cells survived significantly better than scrambled shRNA-transfected cells in response to RA treatment (Fig. 4C).

Collectively, these results suggest that HOXA5 is a direct target of RARβ and is partially responsible for RA-mediated apoptosis and cellular growth inhibition.

Discussion

Previous studies from our laboratory have shown that HOXA5 expression is lost in >60% primary breast carcinomas, and that introduction of the HOXA5 gene into breast cancer cells induced apoptosis through both p53-dependent and p53-independent mechanisms (27, 29). By identifying upstream activators of HOXA5 expression, it might be possible to devise targeted treatment strategies. Based on findings that HOXA5 expression is induced in embryonic stem cells and developing mouse lung by RA (25, 42), we hypothesized that HOXA5 expression is regulated in breast cells by the RARs and/or RXRs. As predicted, we found that HOXA5 expression is induced by RA in the majority of RARβ-positive breast cells. Further, for the first time, we defined the RARE in the HOXA5...
coding region, and showed that among the RARs, RARβ is the critical determinant of induction of HOXA5 and subsequent apoptosis induced by RA.

**HOXA5 is a direct target of RARβ in breast cancer cells.** We identified a specific RARE in the 3’ end of the HOXA5 coding region. The RARE was a direct repeat site with a 3-bp spacing (DR3); the first half site contained a consensus RA binding motif (AGGTCA) to which, potentially, all of RARs can bind. Our transient transfection and ChIP assays together showed that RA induced HOXA5 expression preferably via RARβ.

**Figure 3.** HOXA5 acts directly downstream of RARβ. A, the pLC-A5GF3-Luc plasmid was cotransfected into CV-1 cells with RAR-expressing plasmids (pSHrRARα, pSHrRARγ, and pSHrRARγ), RXRα-expressing plasmid (pSHrRXRα), or vector (pRSV-0). The transfected cells were treated with 1 μmol/L of RA for 24 h before being harvested for luciferase analysis. B, knockdown of RARβ expression blocked the induction of HOXA5 expression by RA. Breast cancer cell line Hs578T cells were transiently transfected with siRNA to RARβ. Then, the cells were treated with RA for 24 h before being harvested for Western blot analysis of HOXA5 and RARβ expression. Quantitative analysis of the Western blot was done using IPLab software. C, examination of the binding affinities of RAR receptors to the site II region in vivo. MCF7 cells were transiently transfected with RAR-expressing plasmids (pSHrRARα-Myc, pSHrRARγ-Myc, and pSHrRARγ-Myc) and vector, and then harvested for ChIP analysis with Myc-Tag antibody. n, no DNA; Cont, control region. D, coordinated loss of HOXA5 and RARβ expression in a series of MCF10A cell model. The expression of HOXA5 and RARβ was examined by real-time PCR analysis in epithelial cell lines—MCF10A, the parental (benign); MCF10A transformed with Ras gene, T1K.C12 (premalignant); and MCFCA.1h (malignant). The mRNA expression level of HOXA5 and RARβ are shown relative to parental MCF10A cells (represented as 100%).

**Role of HOXA5 in RA-induced anticancer effects.** Retinoids are effective inhibitors of breast cancer cell growth. Compared with the relatively ubiquitous expression of RARs in breast cancer cells, RARβ expression is lost in majority of breast cancer cell lines and primary breast carcinomas (33, 43), suggesting that RARβ may play an important part in breast tumorigenesis. Several studies have shown that RARβ behaves as a tumor-suppressor gene in breast cancer and that induction of RARβ mediates RA-induced growth inhibition and apoptosis (12, 15, 44–47). However, the mechanism by which RARβ mediates cellular growth inhibition and apoptosis is largely unknown. In this study, we have shown for the first time that HOXA5 is one of the direct targets of RARβ in breast cancer cells.

Our previous studies have shown that HOXA5 induces apoptosis in a p53-dependent (27), as well as a caspase-dependent, pathway...
It is interesting to speculate that HOXA5 induction in breast cancer cells may bridge the RA signaling pathway and apoptotic signaling pathways. Consistent with this notion, when HOXA5 induction was blunted by shRNA in breast cancer MCF7 cells, RA-induced apoptosis was also significantly blocked.

RA-induced gene expression in the HOX clusters. RA is not only a cancer prevention and therapeutic agent for cancer but is also a key morphogen in vertebrate development. Retinoids induce body axis formation in different animal systems while specifically regulating the HOX genes (48, 49). Although HOX genes have been known to be induced by RA shortly after they were identified in the vertebrate, RAREs have been found only in two genes of HOX paralog group 1 (HOXA1 and HOXB1) and three genes from group 4 (HOXA4, HOXB4 and HOXD4; 21, 22). The RAREs for the first paralog HOX genes are located in the 3′-end of the coding region, whereas the RAREs for the fourth paralog genes are located in the 5′-end of the coding regions (21–24). Despite the fact that few RAREs have been found in HOX genes, the entire cluster of HOX genes is sequentially activated by RA in embryonic stem cells. Several models, including direct and indirect regulation of HOX gene expression by RA, have been proposed to explain this sequential activation phenomenon. In one model, the HOX genes are activated via an RA-responsive “locus control region,” and binding of RA to the RARE leads to opening up the chromatin structure of this region. In a second model, the HOX genes located at 3′-end of the cluster are activated directly via the RARE enhancers; the protein products of these genes activate the next 5′-end genes in the clusters. In a third model, multiple RAREs control the response of HOX genes with differing affinities for RA (50). These models do not necessarily exclude each other. Identification of HOXA5 RARE in this study may support the third model although more RARE sites for other...
HOX genes are yet to be identified. Much remains to be understood regarding the precise role of HOX genes in RA-mediated cell death.

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