Implications of RARβ in the oncogenesis of human lung cancer and breast cancer

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INTRODUCTION

Development of cancer is characterized by the abnormally regulated cell growth and differentiation processes. Because of their profound effects on the growth and differentiation of many different cell types *in vitro* and *in vivo*, retinoids, the natural and synthetic vitamin A derivatives have been widely used as chemopreventive and chemotherapeutic agents for a variety of human cancers such as acute promyelocytic leukemia, cervical cancer, metastatic squamous cancer of the skin and squamous-cell carcinoma of the head and neck (1-3). The preventive and therapeutic effects of retinoids against human lung cancer and breast cancer have been well recognized. Low dietary intake or serum levels of β-carotene are often associated with increased risk of lung cancer and breast cancer (4-6). Vitamin A can inhibit carcinogenesis processes of chemically induced broncogenic squamous cell carcinoma as well as mammary tumor. *In vitro*, RA plays a critical role in regulating the differentiation and proliferation of tracheobronchial epithelial cells and suppressing the squamous differentiation of these cells. In breast cancer cells, retinoids are effective inhibitors of their proliferation.

The effects of retinoids are mediated by two classes of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (7). RARs and RXRs are part of the steroid/thyroid hormone receptor superfamily (8). These receptors function as ligand-activated transcriptional factors that bind to specific responsive sequences on the target genes and regulate the transcriptional expression of these genes (9, 10). Both types of receptors are encoded by three distinct genes, α, β and γ. All-*trans* retinoic acid (RA) and 9-*cis* RA, the two known active derivatives of Vitamin A, essentially function as hormones by interacting with specific retinoid receptors that bind to RA response elements (RAREs) and thereby control the expression of RA responsive genes.

The cancer preventive and therapeutic effects retinoids are likely mediated by their nuclear receptors. Alterations in the transcriptional activities of retinoid receptors, therefore, could results in abrogation of RA-dependent gene expression and phenotypic changes in retinoid target cells. The association between altered RAR transcriptional activities and carcinogenesis was demonstrated in many cancer cell types including acute promyelocytic leukemia, liver cancer, and oral squamous cell carcinoma cell lines (11). Although the detail mechanism by which a loss of RARβ gene expression contributes to cancer development is unclear, the observations from the present study together with others (11-13), suggest that an abnormal expression of RARβ gene may be an important contributing factor in the development of human lung cancer and breast cancer.

RESULTS and DISCUSSION

Abnormal expression of RARβ gene in human lung cancer and associated mechanisms.

To establish the involvement of retinoid receptors in mediating retinoid response in

lung cancer cells and lung cancer development, the expression of retinoid receptor genes in various lung cancer cell lines, including small cell carcinoma (H146), squamous cell carcinoma (SK-MES-1), adenosquamous carcinoma (H292), large cell carcinoma (H661), adenocarcinoma (Calu-3), and anaplastic carcinoma (Calu-6) was examined by Northern blot analyses (14). Similar expression levels of RARα and RARγ as well as RXRα and RXRβ genes were detected in all these cell lines (Figure 1 and data not shown). However, the expression levels of RARβ gene varied significantly and, more importantly, in most of lung cancer cell lines, RARβ expression is no longer regulated by RA, suggesting that lack of the RARβ gene expression may be involved in the development of lung cancer.

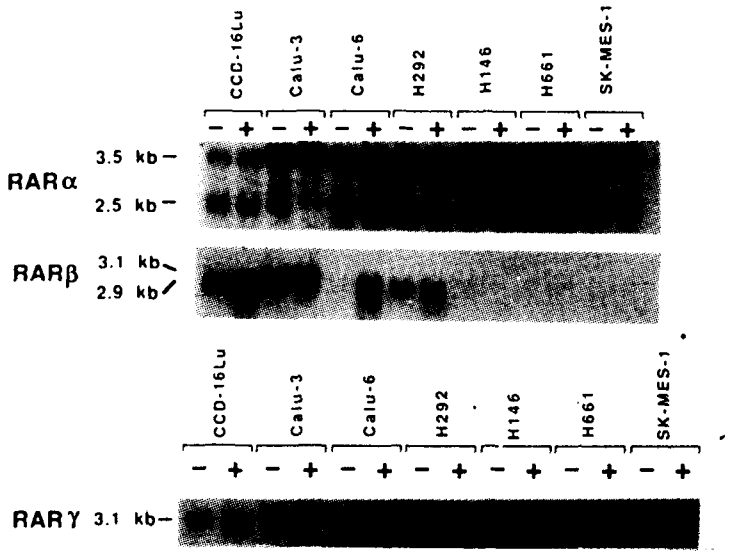

Figure 1. Expression of retinoid receptors in the human lung cancer cell lines. *Different types of human lung cancer cells were grown in the presence or absence of RA* (10^{-6} M). Total RNA (20 μg) prepared from different cultures was separated, transferred to Nylon filters, probed with the P^{32}-labeled ligand-binding domain of the indicated receptors. kb. Kilobases.

RA-induced RARβ expression is mediated by the βRARE present in RARβ promoter. To test whether the loss of the RA response is due to abnormal transcriptional regulation of the RARβ gene promoter, the RARβ promoter was linked to the CAT gene. The resulting reporter construct was transiently transfected into both RA-responsive (Calu-6) and non-responsive (H661, SK-MES-1, and H292) cancer cell lines. In RA-responsive Calu-6 cells, induction of CAT activity was observed in the presence of RA (Figure 2A). In RA-non-responsive cell lines, variable results were obtained. The RARβ gene promoter was strongly induced by RA in H661 cells, while treatment with RA did not clearly induce CAT activity in SK-MES-1 and H292 cells (Figure 2A). To examine the activity of the βRARE in the lung cancer cell lines, a CAT reporter construct containing the βRARE linked to the heterologous tk promoter (βRARE-tk-CAT) was introduced into cancer cell lines with both RA-responsive and -non-responsive RARβ genes. Surprisingly, in contrast to the results obtained with the RARβ gene promoter reporter, induction of the βRARE-tk-CAT gene in response to RA was also observed in SK-MES-1 and H292 cells (Figure 2B). These results suggest that these lung cancer cell

lines contain all necessary receptors to activate the βRARE.

Although the mechanism of the abnormal regulation of the RARβ gene is currently unclear, our results suggest that multiple mechanisms may exist. First, factors, other than RARs and RXRs, may be required to mediate the transactivation function of RARs and RXRs in the RARβ gene promoter. These factors appear to be missing or mutated in SK-MES-1 cells. Although the nature of these factors is presently unknown, it has been suggested that retinoid receptors require co-activator(s) for efficient transactivation activity. E1A like protein has been reported to function as co-activator of RA-dependent RARβ promoter transactivation (15). Recently, Moghal and Neel suggested the existence of novel RAR-thyroid hormone receptor AF-2 specific cofactors, which are necessary for high levels of transcription (13). It is expected that these unknown RXR-interacting proteins which function as retinoid receptors co-activators on the βRARE likely play an important role in mediating RA responses, and that lack or alteration of these proteins may be responsible for the impaired RA response in inducing RARβ gene expression in some lung cancer cell lines. Second, the observations that no detectable expression of RARβ gene in H661 cells either in the absence or presence of RA (Figure 1) while cotransfected RARβ promoter reporter or βRARE-tk-CAT was activated in response to RA, suggest that the loss of RA response observed in this cell lines may be due to, most likely, a mutation(s) in the endogenous RARβ promoter. Finally, we observed a strongly reduced RA response on a βRARE-tk-CAT reporter in H292 cells, which may not be biologically significant. Since endogenous RARs and RXRs are highly expressed in H292 cells (Figure 1), the loss of the βRARE activity in H292 cells likely due to mutations present in endogenous retinoid receptors. Mutations within nuclear receptor genes have been found in many different diseases and are associated with their diminished ligand responsiveness (16, 17). For instance, in patient with generalized resistance to thyroid hormone, single

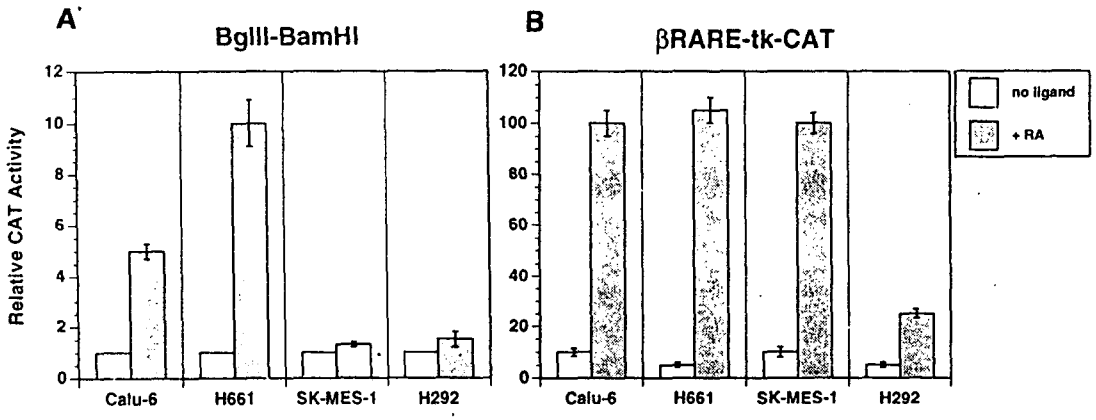

Figure 2. Transcriptional activity of the RARβ gene promoter and βRARE in human lung cancer cell lines. *A.* Transcriptional activity of BglII-BamHI (-745 to +156) fragment of RARβ gene promoter. BglII-BamHI -BS-CAT (2.0 μg) and β-gal expression vector (3.0 μg; pCH110) were transiently transfected into the indicated cells and were grown in the presence or absence of RA (10^{-7} M). *B.* Activity of the βRARE in lung cancer cell lines. The βRARE linked to the heterologous tk promoter was examined. βRARE-tk-CAT (2.0 μg) together with the β-gal expression vector (3.0 μg) were transiently transfected into the indicated cells and were grown in the presence or absence of RA (10^{-7} M). Transfection efficiency was normalized by β-gal activity. The activity of the reporter genes in the absence of RA was chosen as the reference value, and CAT activity was normalized accordingly. Bars. SD.

amino acid mutations of TR results in the expression of receptors with either reduced or no T3 binding activity. Similarly, a single amino acid change in the ligand binding domain of RARα dramatically alters its ligand binding and transcriptional activity. In homology with the above examples, H292 cells may contain mutated retinoid receptor(s) that may have lost their ligand responsiveness.

RARβ mediates the growth inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer

Retinoids are effective inhibitors of the proliferation of hormone-dependent but not of hormone-independent breast cancer cells. The involvement of retinoid receptors in the differential growth inhibitory effect of retinoids and the underlying mechanism have been studied in the present study. When the expression of the retinoid receptor genes was examined) in a hormone-dependent (ZR-75-1 and T-47D) and hormone-independent (MB468, MB231 and BT-20) cell lines, transcripts for RARα and RARγ were detected in all the cell lines with some variations in expression levels, but transcripts for RARβ were not detectable. However, in the presence of RA, the expression of RARβ was strongly enhanced in the hormone-dependent but not in the hormone-independent lines tested which correlates with the growth inhibitory effects of RA (Figure 3). Since hormone-independent breast cancer cells are usually considered to represent those at late stage of breast tumor progression, these observation suggest that the lack of RARβ induction may have important pathogenic consequences during the development of human breast cancer and may be responsible for the diminished retinoid sensitivity during the progression of breast cancer.

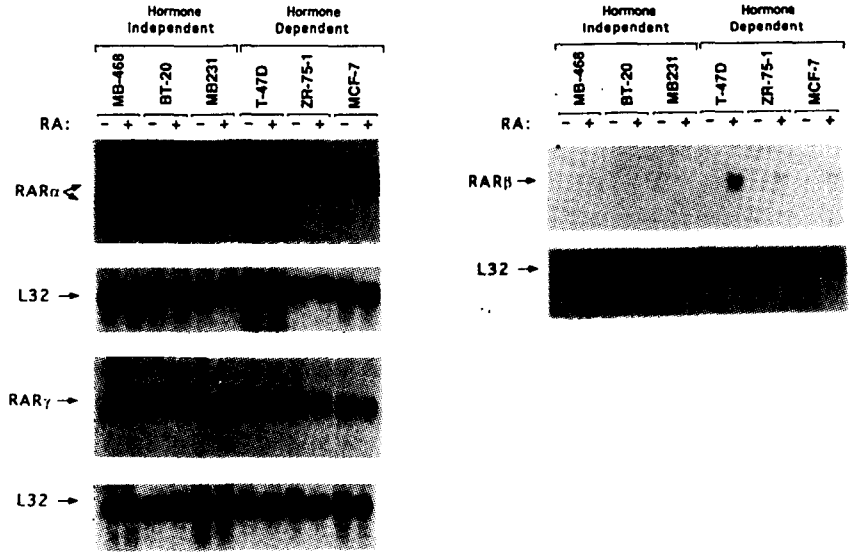

Figure 3. Expression of receptors in hormone-dependent and -independent human breast cancer cells. The expression of RAR genes was determined by Northern blot analysis using total RNA (30 μg) prepared from different breast cancer cell lines. To determine the effect of RA, cells were treated with 10^{-6} M RA for 36 hrs before RNA preparations. The expression of L32 gene, a RA-independent gene, was monitored as a control.

To obtain direct evidence that the loss of RA response in hormone-independent breast cancer cells is due to insufficient levels of RARβ expression, RARβ encoding vector was stably transfected into hormone-independent cells, *i.e.*, MB231 (Figure 4). Two clones obtained that expressed exogenous RARβ, *i.e.*, MB231/RARβ2 and MB231/RBRβ3, acquired RA sensitivity in the growth inhibition assays while the growth of parental MB231 or MB231 cells transfected with empty vector, *i.e.*, MB231/vector, was not significantly affected, demonstrating that the expression of RARβ is essential for RA-induced growth inhibition in hormone-dependent breast cancer cells.

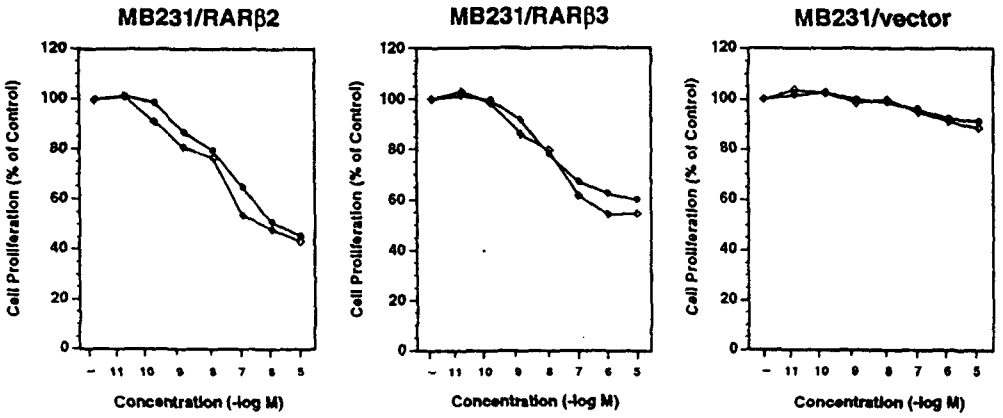

Figure 4. Stable expression of RARβ gene restores RA sensitivity in hormone-independent breast cancer cells, MB231. To construct RARβ expression vector, RARβ cDNA was cloned into pRc/CMV expression vector. The resulting pRc/CMV-RARβ recombinant construct was stably transfected into MB231 cells by calcium phosphate precipitation method and screened using G418. The integration and expression of exogenous RARβ were determined by Northern blot analyses. Two thousand cells per well were seeded and treated with the indicated concentrations of retinoids for 7 days. The number of viable cells were determined by their capacity to convert a tetrazolium salt into a blue formazan product using a non-radioactive Cell Proliferation/Cytotoxicity Assay Kit (Promega, Madison, WI). Filled circle represents all-*trans*-RA treatment and open diamond indicates 9-*cis*-RA treatment.

When RARβ was expressed in MB231 cells, we noticed that many RA-treated cells detached and became shrunken, followed by cell death. When the nuclei of these cells stained by propidium iodine (PI) and examined by fluorescence microscopy, we found that many of the RA-treated MB231/RARβ3 cells were smaller and contained fragmented nuclei with brightly staining chromatin, which are morphological changes typical of apoptosis (18). RA caused similar morphological alterations in the RA-sensitive lines ZR-75-1, MCF-7 and T-47D but not in the RA-resistant lines, MB231, MB-468 and BT-20. Furthermore, DNA fragmentation indicative of apoptosis was also detected in ZR-75-1 and MB231/RARβ3 cells by end-labeling of the 3'OH ends of fragmented DNA by terminal deoxynucleotidyl transferase (TdT). Interestingly, ZR-75-1 cells that expressed RARβ anti-sense RNA experienced significantly less apoptotic process (Figure 5). Together, these data demonstrate that RA can induce apoptosis in RA-sensitive breast cancer cells and suggest that the RA-induced apoptotic process is likely mediated, in part, by RARβ.

CONCLUSIONS

we demonstrated that lack of RARβ gene expression and abnormal regulation by RA is a common feature associated with both human lung and breast cancer. We further showed that the hormone-independent breast cancer cells acquired RA sensitivity when RARβ expression vector was introduced and expressed in the cells. Induction of apoptosis contributes, at least in part, to the growth inhibitory effect of RARβ. These observations imply that abnormal expression and regulation of RARβ gene may contribute to cancer development and affect tumor progression. A better understading of the mechanism by which the expression of RARβ gene is regulated will help restoring functional RARβ activities in cancer cells.

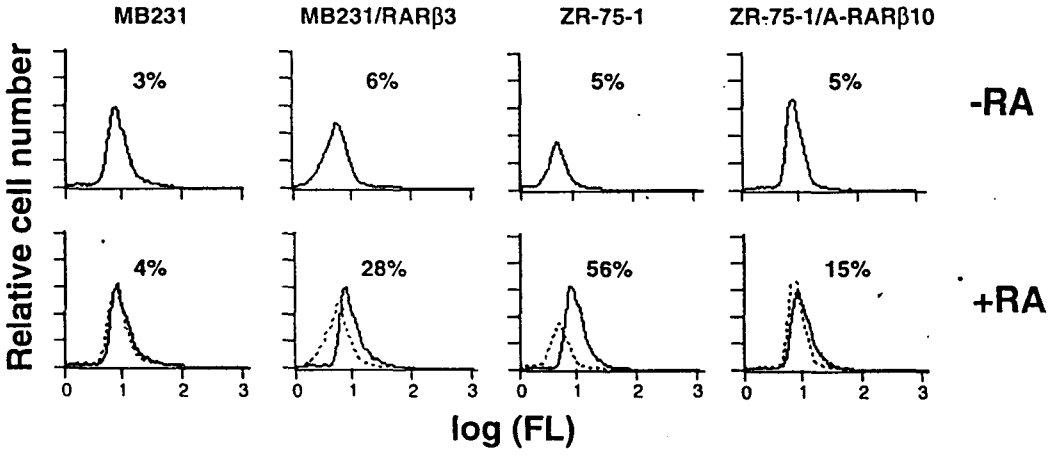

Figure 5. RARβ promotes cell Apoptosis. TdT-mediated dUTP-X nick end labeling (TUNEL) assays of parental and ectopic RARβ transfected MB231 cells, and parental and antisense RARβ transfected ZR-75-1 cells after 24 hrs of RA treatment. Cells were trypsinized, washed with PBS, and fixed in 1% formaldehyde in PBS (pH 7.4). After washing with PBS, cells were resuspended in 70% ice-cold ethanol and immediately stored at -20°C for overnight. Cells were then labeled with biotin-16-dUTP by terminal transferase and stained with avidin-FITC (Boehringer Mannheim, Germany). Fluorescence labeled cells were analyzed using a FACStar-Plus. Representative histograms are shown here.

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