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INVOLVEMENT OF RETINOIC ACID NUCLEAR RECEPTORS IN RETINOIC ACID-INDUCED TISSUE TRANSGLUTAMINASE GENE EXPRESSION IN RAT TRACHEAL 2C5 CELLS[1]

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SUMMARY: The involvement of retinoic acid nuclear receptors (RARs) in the induction of tissue transglutaminase (TG) by retinoic acid in rat tracheal 2C5 cells was determined. The levels of RARα and RARβ were altered in 2C5 cells by transfection with RAR expression vectors. Increased expression of RARα increased the induction of tissue TG by retinoic acid. In contrast, decreased RARα expression, using an antisense RARα expression vector, diminished the normal level of tissue TG induction caused by retinoic acid. Transfectants overexpressing RARβ were also more responsive to retinoic acid for the induction of tissue TG, although the magnitude of TG induction was not as great as resulted from RARα overexpression. These results indicate that the levels of the RARα and RARβ dictate the magnitude of tissue TG induction by retinoic acid. © 1991 Academic Press, Inc.

Retinoic acid (RA), a major metabolite of vitamin A, has been shown to regulate the expression of a variety of genes, including tissue transglutaminase (TG) (1, 2). TG belongs to a family of enzymes involved in the post-translational modification of proteins by catalyzing covalent ε(γ-glutamyl)lysyl crosslinks (4, 5). The induction of tissue TG mRNA occurs early following RA treatment and is mediated at least in part by increased transcription of the tissue TG gene (3). The exact molecular mechanism of the induction of tissue TG by RA remains unclear. However, it has been hypothesized to be interceded by one or more of a family of nuclear retinoic acid receptors (RARs) which act as ligand-inducible transcription factors (3). To date, several human retinoic acid receptors (i.e., α, β, γ and RXR) have been identified (6-11), however, their individual roles in regulating gene expression are unknown.

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We characterized the rat tracheal cell line 2C5 to analyze the mechanism by which RA modulates gene expression and differentiation (12, 13). 2C5 cells provide an excellent model for the investigation of RA action because these are epithelial cells which undergo squamous differentiation that can be reversed by retinoic acid treatment. We found that treatment of 2C5 cells with RA results in dramatic induction of tissue TG activity. To determine if either retinoic acid receptor α (RARα) or retinoic acid receptor β (RARβ) is involved in the regulation of tissue TG gene expression, we modulated the levels of RARs in 2C5 cells by using transfection techniques. Data indicating that the level of expression of RARα or RARβ in 2C5 cells is correlated with the degree of induction of tissue TG by RA are summarized in this communication.

MATERIALS AND METHODS

Materials

RA was purchased from Sigma Chemical Company, St. Louis, MO. [2,3-^{3}H(N)]putrescine dihydrochloride (15-30 Ci/mmol) was purchased from New England Nuclear, Boston, MA. The human retinoic acid receptor expression vectors RAR-αØ and RAR-βØ were kindly provided by Dr. Pierre Chambon, Strasbourg-Cedex, France. The RARα antisense expression vector RAR-AαØ was constructed by reversing the orientation of the EcoRI fragment containing the RARα cDNA. pSV2-Neo was purchased from American Type Culture Collection, Rockville, MD. The tissue transglutaminase cDNA containing plasmid pTG9.9.1 was kindly provided by Dr. Joseph P. Stein, Houston, TX. The 2C5 cell line was obtained from Dr. Vernon E. Steele, Research Triangle Park, NC.

Cell Culture

Rat tracheal 2C5 cells were grown in Waymouth's MB752/1 media containing 5% fetal bovine serum. The media also contained the following supplements: 50 u/ml penicillin, 50 mg/ml streptomycin, 0.0178 mg/ml L-alanine, 0.347 mg/ml L-arginine, 0.021 mg/ml L-serine, 0.03 mg/ml L-asparagine, 0.22 mg/ml Na pyruvate, 0.01 mg/ml insulin, 0.005 mg/ml transferrin, 10^{-6} M hydrocortisone, and 0.1% endothelial cell growth supplement.

Transfections and Selection of Transfectants

Cells were transfected with the expression vectors RAR-αØ, RAR-βØ or RAR-AαØ plus pSV2-Neo (5:1 ratio) by the calcium phosphate method as previously described (14). Transfectants were grown and expanded in media containing 400 μg/ml G418 until they were frozen and harvested for DNA and RNA isolation.

Southern and Northern Blots

DNA and RNA were isolated from approximately 10^7 cells as described by Gough (15). RNAs of interest were detected by a modification of the Northern blot procedure described by Thomas (16). The Southern blot procedure was adapted from the technique described by Southern (17).

Transglutaminase Assay

Transglutaminase was assayed by quantitating the incorporation of [2,3-^{3}H(N)]putrescine dihydrochloride into N,N-dimethyl casein according to the method of Lichti *et al.* (1).

RESULTS

The effect of RA on tissue TG activity in 2C5 cells is illustrated in Figure 1. In this experiment, 2C5 cells were cultured for 4 days in

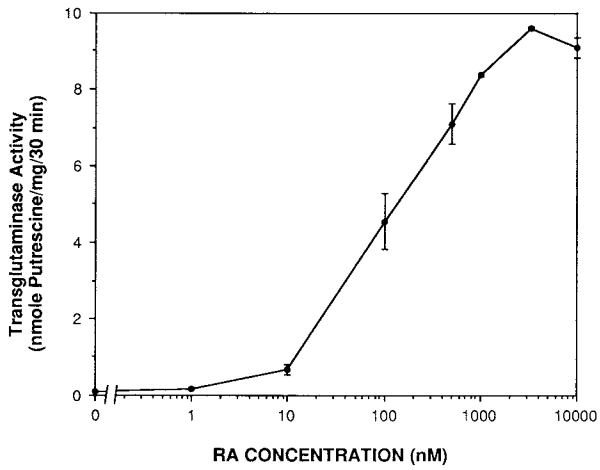


Figure 1. Effect of RA on tissue TG activity in 2C5 cells. Soluble (tissue) TG activity was assayed 4 days after culturing 2C5 cells in the presence of the indicated concentration of retinoic acid. Each value is the mean ± standard deviation of determinations from two culture dishes.

the presence of various concentration of RA, and tissue TG activity was assayed. TG activity was induced by RA in a dose-dependent manner, with half maximal induction at approximately 100 nM RA. The maximum induction of about 110 fold was observed at 3.3 μM RA.

To alter the level of RARα and RARβ, we transfected 2C5 cells with RARα and RARβ expression vectors (RAR-αØ and RAR-βØ, respectively) and an RARα antisense expression vector (RAR-AαØ). The presence of the transfected DNA in several transfectants was verified by Southern blot (Figure 2). As shown in Figure 2A, the DNA from several of the RAR-αØ

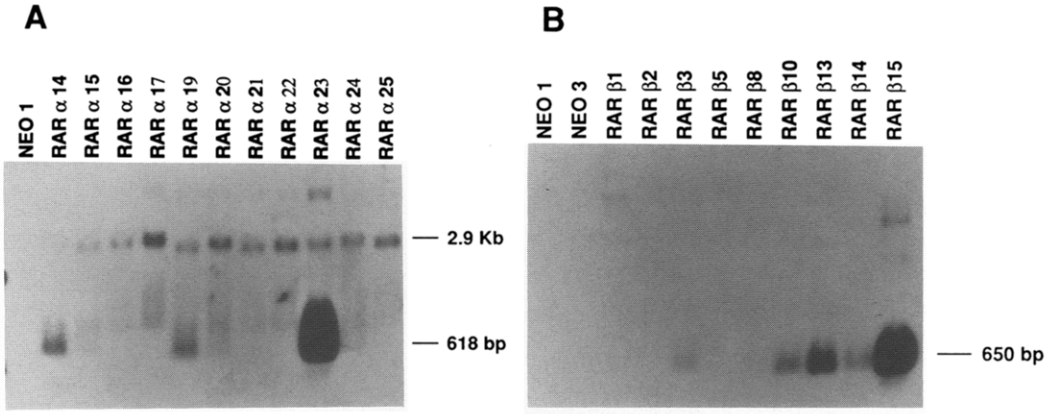


Figure 2. Southern blot analysis of RAR transfectants.

A: 10 μg of DNA from cells transfected with either pSV2-Neo (Neo1) or pSV2-Neo and RAR-αØ (RARα14 to RARα25) was digested with PstI and subjected to Southern blot analysis on a 1% agarose gel. The DNA was probed with the 618 bp Pst I fragment of RAR-αØ.
B: 10 μg of DNA from cells transfected with either pSV2-Neo (Neo1 and Neo3) or pSV2-Neo and RAR-βØ (RARβ1 to RARβ15) was digested with EcoR I and the Southern blot probed with the 652 and 615 bp EcoR I fragments from RAR-βØ.

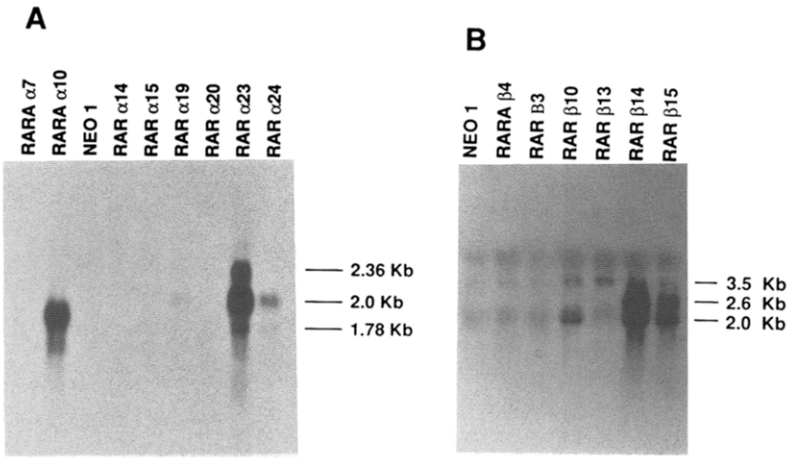


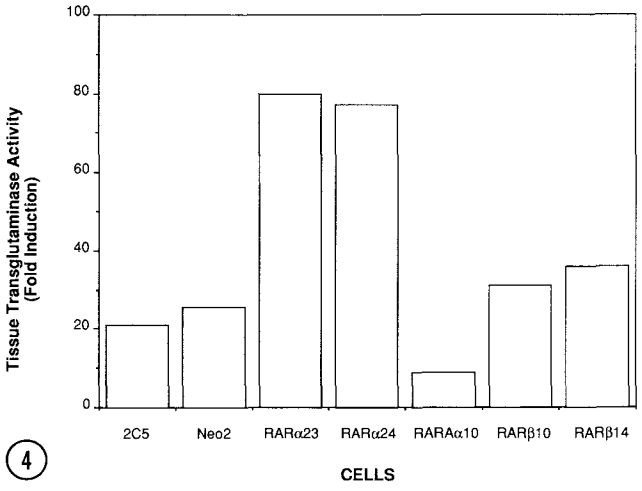
Figure 3. Northern blot analysis of RAR transfectants.

A: 10 µg total RNA from cells transfected with either pSV2-Neo and RAR-AαØ (RARAα7 and RARAα10), pSV2-Neo only (Neo1), or pSV2-Neo and RAR-αØ (RARα14 to RARα24) was loaded per lane and probed with the 1.78 kb EcoR I fragment from RAR-αØ.
B: 10 µg total RNA from cells transfected with either pSV2-Neo (Neo1) or pSV2-Neo and RAR-βØ (RARβ3 to RARβ15) was loaded per lane and probed with the 652 bp and 615 bp EcoR I fragments of RAR-βØ. RARAβ4 was transfected with pSV2-Neo and a RARβ antisense expression vector RAR-AβØ. The blots were washed to high stringency and no cross-hybridization was detectable.

transfectants contained a 618 bp Pst I fragment which hybridized to RARα. This 618 bp fragment is the same size as the Pst I fragment from the transfected expression vector and was absent from the Neo1 transfectant. The RARα cDNA copy number was estimated by laser beam densitometry and varied from 1 for RARα24 to approximately 60 for RARα23. The 2.9 kb band represented the endogenous RARα gene since it was present in all the transfectants at approximately equal intensities. Southern blot analysis of the RAR-βØ transfectants is shown in Figure 2B. The 652 bp EcoRI fragment hybridized to RARβ was present only in the RAR-βØ transfectants and absent in the pSV2-Neo transfectants.

The level of RAR expression in the transfectants was quantitated by Northern blot analysis. As shown in Figure 3A, RARα was overexpressed in the transfectants RARα23 and RARα24. The antisense transfectant RARAα10 was expressing very high levels of antisense RNA. The RARα transcript sizes were 2.36 kb, 2.0 kb and 1.78 kb and may have resulted from differences in splicing and/or polyadenylation. Figure 3B illustrates that the RARβ transfectants RARβ10, RARβ13, RARβ14, and RARβ15 also overexpressed the transfected RAR. The sizes of the RARβ transcripts were 3.5 kb, 2.6 kb and 2.0 kb.

TG was induced by RA in several of the transfectants to determine if the degree of induction of TG correlated with the level of RAR. While TG activity was induced approximately 21-fold by 100 nM RA in the parental 2C5 cells, the RARα overexpressing transfectants RARα23 and RARα24 induced TG activity by 80- and 77-fold, respectively (Figure 4). TG activity in the RARα antisense transfectant, RARAα10, was increased by approximately



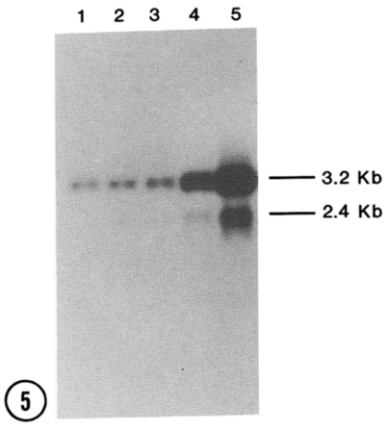


Figure 4. Effect of RA on tissue TG activity in 2C5 cells and transfectants. The indicated transfectants and parental 2C5 cells were grown either in the presence or absence of 100 nM RA and tissue TG activity assayed after 4 days treatment.

$$\textit{Fold induction} = \frac{\textit{TG activity in the presence of RA}}{\textit{TG activity in the absence of RA}}$$

Figure 5. Effect of RA on tissue TG mRNA in RARα23 cells. Cells were grown for 4 days in the presence of different RA concentrations and then RNA was isolated. Shown is the autoradiogram of Northern blot analysis. 10 μg total RNA per lane was loaded and probed with the tissue TG cDNA containing 1.6 kb EcoR I fragment from pTP9.9.1. Treatments were as follows: lane 1, ethanol; lane 2, 0.1 nM RA; lane 3, 1.0 nM RA; lane 4, 10 nM RA; lane 5, 100 nM RA.

9-fold. RA, at 100 nM concentration, stimulated TG activity in RARβ transfectants RARβ10 and RARβ14 by 31-fold and 36-fold, respectively. The induction of TG activity in the pSV2-Neo transfectant Neo2 was similar to that of the parental 2C5 (~25-fold).

To determine if the increase in TG activity by RA was truly the result of increased gene expression and not a posttranslational modification or activation of the enzyme, the level of TG mRNA was determined by Northern blot analysis. The results are shown in Figure 5. The induction of TG activity in RARα23 cells was accompanied by a proportional increase in the steady-state level of TG mRNA. RA treatment caused a dose-dependent increase in two tissue TG transcripts of sizes 3.2 kb and 2.4 kb. The 3.2 kb message was approximately 5 times more abundant than the 2.4 kb message.

DISCUSSION

RA has been proposed to mediate its diverse effects on differentiation and development by altering gene expression (18-21). However, a precise mechanism for the regulation of RA-responsive genes still remains speculative (22, 23). Now, we present direct evidence indicating the involvement of RARs in RA-induced TG expression in 2C5 cells.

Tissue TG is induced by RA in mouse peritoneal macrophages (3), mouse epidermis (2) and PC12 pheochromocytoma cells (24). We also found a

dramatic induction of tissue TG activity after treatment of rat tracheal 2C5 cells with RA (Figure 1). Since 2C5 cells contain low but detectable levels of RARα, RARβ and RARγ mRNA (data not shown), we explored the possibility that one or more of these retinoic acid receptors are responsible for the induction of TG by RA. Clearly, the induction of tissue TG by 100 nM RA (ED_{50} concentration) was greater in the RARα overexpressing transfectants. The role of RARα in regulating the induction of TG by RA levels was further strengthened by the finding that the antisense transfectant RARAα10 was not as responsive as the parental 2C5 cells and pSV2-Neo transfectants. These results are consistent with the findings that the level of RARα determines tissue TG induction by RA (Figure 4).

Both RARβ overexpressing transfectants (RARβ10 and RARβ14) were more responsive than parental 2C5 cells to RA for the induction of tissue TG. However, RARα overexpressing transfectants were more responsive than RARβ transfectants. These results indicate that RARα may be more important than RARβ in the regulation of TG gene expression in 2C5 cells by RA. Since the relative abundance of RARα and RARβ proteins in the transfectants are not known, this difference in the induction of TG activity could also be due to differences in the levels of RARα and RARβ in the overexpressing transfectants.

The induction of tissue TG activity was accompanied by an increase in two TG mRNAs of 3.2 and 2.4 kb in size (Figure 5). The induction of TG mRNA was correlated with an increase in TG activity caused by RA ($r = 0.999$). These results indicate that RA-induced TG activity is the result of increased TG gene expression.

In summary, results (Figure 4) presented clearly indicate that modulation of the levels of expression of either RARα or RARβ by transfection of 2C5 cells with sense or antisense RAR expression vector accordingly modulated the ability of RA to induce TG gene expression. Thus, one may conclude that RARα and RARβ are critical components of the RA signal transduction pathway regulating tissue TG gene expression.

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