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RETINOIC ACID RECEPTOR TRANSCRIPTS IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Laszlo Fesus[1], Laszlo Nagy[1], James P. Basilion[2] and Peter J. A. Davies[2]*

[1]Department of Biochemistry, University School of Medicine, Debrecen, Hungary

[2]Department of Pharmacology, The Medical School, University of Texas Health Sciences Center at Houston, Houston, Texas

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Summary Human umbilical vein endothelial cells contain high levels of mRNA for the β-retinoic acid receptor,and very low levels of α-retinoic acid receptor transcripts. The cells responded to retinoic acid with a significant induction of tissue transglutaminase expression but no alterations in the expression of β-retinoic acid receptor transcripts. The physiological implications of the constitutive expression of this receptor in endothelial cells is discussed.

Introduction Retinoids have long been recognized as physiological regulators of angiogenesis and endothelial cell function. Inadequate supplies of dietary retinol have been associated with vascular hypoplasia in the chick embryo (1) and both retinol and retinoic acid have been shown to have anti-angiogenic activity in vivo (2). These growth inhibitory effects have been linked to an ability of retinoids to suppress mitogen stimulated proliferation of cultured endothelial cells (3). In addition retinoids can induce morphologic alterations and changes in the pattern of gene expression and protein synthesis in endothelial cells cultured in vitro (3-7). In spite of the diverse effects of retinoids on endothelial cell biology, little is known of the molecular basis of these effects. Recently several laboratories have shown that the effects of retinoids on many cellular functions are mediated by a family of retinoic acid receptors (8-12). The studies reported here were designed to characterize the retinoic acid receptors expressed in cultured human umbilical vein endothelial (HUVE cells).

*To whom reprint requests should be addressed.

Abbreviations: HUVE: human umbilical vein endothelial; EDTA: ethylenediamine tetraacetic acid; RAR: retinoic acid receptor; EtBr: ethidium bromide; Cyclic AMP: cyclic-3′-5′-adenosine monophosphate; SDS: sodium dodecyl sulfate.

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Materials and Methods Primary cultures of human umbilical vein endothelial (HUVE) cells were prepared and maintained in culture on gelatin coated dishes in medium 199 supplemented with 20% fetal calf serum (GIBCO, Grand Island, N.Y.), endothelial growth factor (Biomed Technologies, Stoughton, MA, 50 μg/ml), heparin (100 μg/ml) and antibiotics (13). The cells were passaged at a 1:3 split ratio twice a week and used in experiments following their third passage. Confluent cells were washed three times with cold Tris-buffered saline, pH7.4, detached with a buffer composed of Tris-buffered saline, EDTA (5 mM) and then were centrifuged at 1000 x G at 4° C. Cell extracts were prepared using 0.5% triton X-100 and transglutaminase activity was assayed in the presence of 5 mM Ca+2 by measuring the incorporation of ^{3}H-putrescine New England Nuclear, Boston, MA) into dimethyl casein (14). The protein concentration of the cell extract was measured by a Coomassie Blue binding assay (15).

For Northern blot analysis (16), RNA was extracted from HUVE cells by the guanidine isothiocyanate /CsCl method (17) and was then fractionated on a formaldehyde / agarose gel. The gel was first stained with EtBr to visualize ribosomal RNA, nucleic acids were transferred to nylon filters and the immobilized RNA's were hybridized to radiolabelled cDNA probes at 42° C in the presence of 25% formaldehyde. These probes were random-primed ^{32}P-labelled cDNA's corresponding to the ligand binding domain of either the human α-RAR (662 bp Sac I/ Pst I restriction endonuclease fragment) or the human β-RAR (615 bp Eco R1 restriction fragment). The human α-RAR and β-RAR cDNA clones were gifts from Drs. R. Evans (La Jolla, CA) and P. Chambon (Strasbourg, France) respectively.

An oligo - dT and random primed cDNA library was constructed from poly A^{+} RNA from HUVE cells inserted into the lambda ZAP phage expression vector (Stratagene, La Jolla, CA.). E Coli strain XL-1 Blue Cells (Stratagene, La Jolla, CA.) were infected with recombinant phage and plaques were screened with random primed ^{32}P-cDNA probes spanning the ligand binding domains (see above) of either the human α-RAR or β-RAR. Filters were hybridized to the probes overnight at 42° C in a buffer containing 25% formamide using standard conditions described by Sambrook et al. (16). The filters were washed extensively with the final was at 55° C in 0.1% SSC / 0.1% SDS. Following biological excision and rescue, positive plasmids were analyzed by restriction mapping, Southern blot analysis (16) and partial sequencing with the dideoxy chain termination method (18).

Results To isolate cDNA clones for the human endothelial cell retinoic acid receptors, a cDNA library prepared from HUVE cell mRNA was screened with hybridization probes from the ligand binding domains of the human α-RAR (662 bp Sac I to Pst I fragment) and the human β-RAR (615 bp Eco R1 fragment). Eight clones, identified by low stringency hybridizations (42° C with 25% formamide - see Materials and Methods), were plaque purified and characterized by restriction endonuclease mapping and DNA sequence analysis (Figure 1). Four clones contained sequences identical to the sequences of the human β-RAR and one clone contained sequence identical with the sequence of the human α-RAR. The two other clones contained sequences that were similar but not identical to the human RAR's (data not shown).

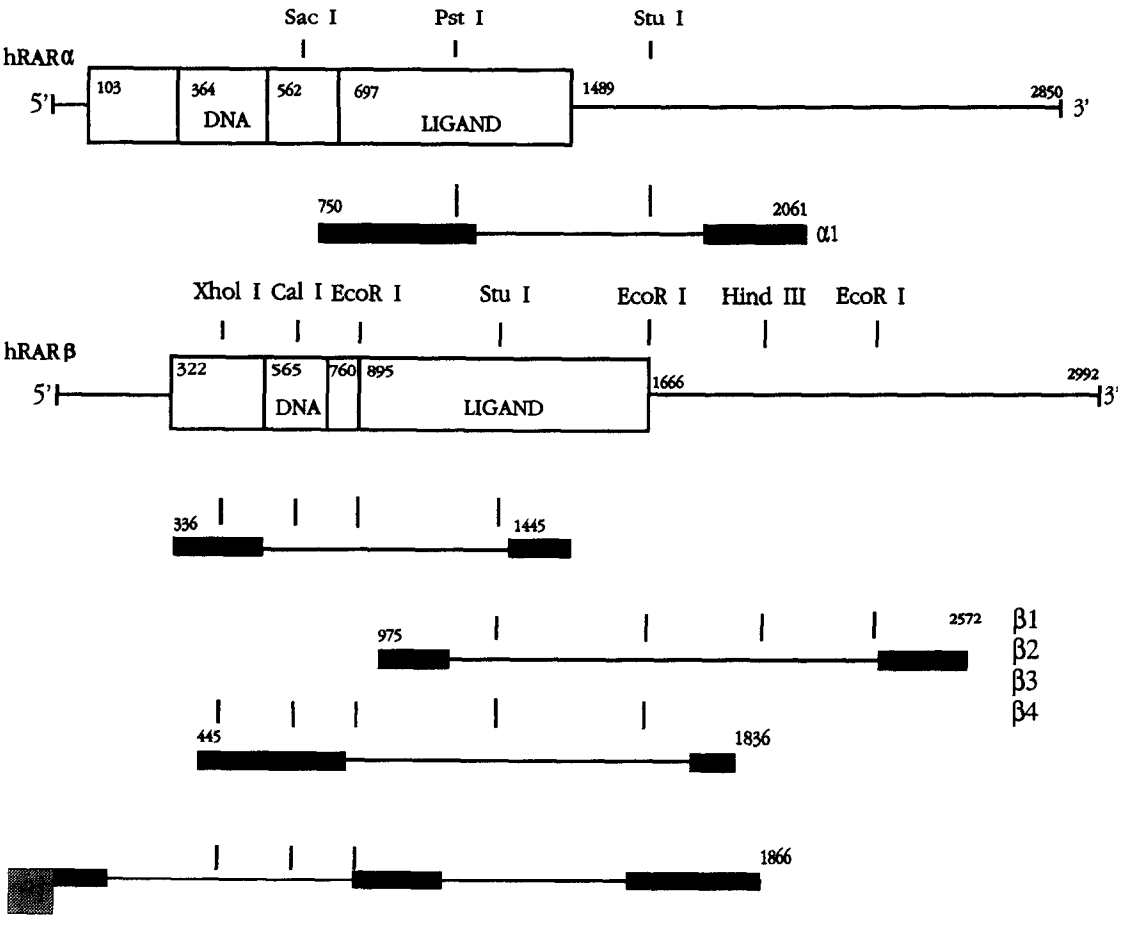
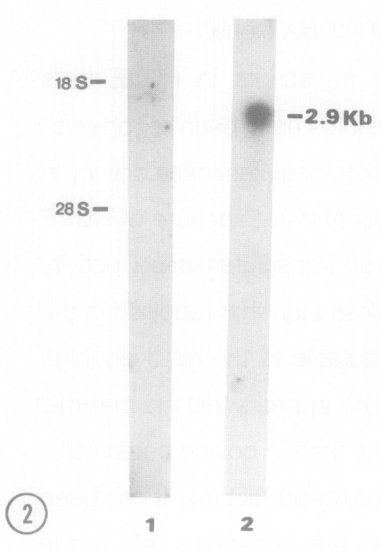


Fig.1. Schematic representation of the restriction sites and partial DNA sequences of retinoic acid receptor cDNA clones isolated from a HUVE cell cDNA library. The open boxes represent the functional domains of the human α- and β- retinoic acid receptors, the filled boxes represent regions of the endothelial cell cDNA clones that have been sequenced and found to be identical with either the human α-RAR (HUVE α1) or β-RAR (HUVE β1,2,3 and 4). The numbers indicate nucleotide positions and the arrows restriction endonuclease - sensitive sites.

The cloning studies suggested that β-RAR's were the predominant retinoid receptor in HUVE cells. To estimate the level of expression of RAR transcripts, total RNA from HUVE cells was fractionated by agarose gel electrophoresis and blots of the RNA were subject to Northern blot analysis with DNA probes prepared from the ligand binding domain of either the α- or β-RAR's (Figure 2). A single, abundant 2.9 kB transcript was readily detected in HUVE cell RNA probed with the β-RAR probe, no α-RAR transcripts were detected by hybridization to the α-RAR probe. Exposure of the HUVE cells to retinoic acid (1 μM) for 24 h resulted in no change in the level of β-RAR mRNA (data not shown). To determine whether the HUVE cells were retinoic acid - responsive, cells were treated for 24 h with retinoic acid (1 μM) and the level of tissue transglutaminase



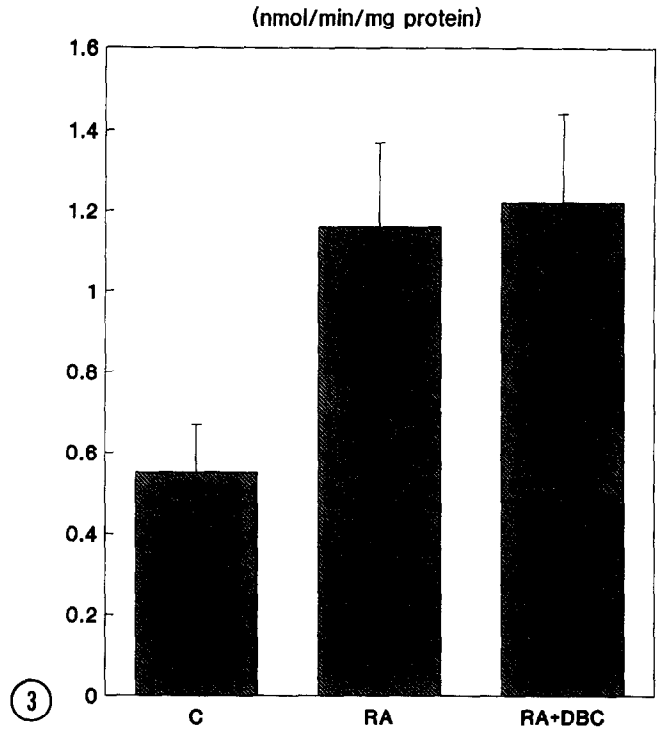


Fig. 2. Northern blot analysis of retinoic acid receptor transcripts in HUVE cell RNA. Total RNA (15 μg) from HUVE cells was fractionated, blotted and hybridized to radiolabelled cDNA probes derived from the ligand binding domain of the human α-RAR (Lane 1) or the human β-RAR (Lane 2).

Fig. 3. Transglutaminase activity in HUVE cell extracts. HUVE cells were exposed to media alone (C) or media containing either 1 μM retinoic acid alone (RA) or retinoic acid plus 1 mM dibutyryl cyclic AMP (RA + DBC) for 24 h. Cells were homogenized and the transglutaminase activity was determined. Values represent the mean and standard error of 5 experiments.

activity was determined by enzymatic assay (Figure 3). Retinoic acid induced a 2-fold increase in the transglutaminase activity of the cells, this effect was unmodified by addition of a cyclic AMP analogue (1 mM dibutyryl cAMP).

Discussion Retinoids have been shown to induce striking effects in the growth and differentiation of many cells (19) including endothelial cells (3-7,20,21). Many of the biological effects of retinoids are thought to be mediated via interactions with one of three retinoic acid receptors, RAR-α,-β and γ (8-12). RAR-γ transcripts appear to be restricted to the skin and embryonic tissues (8) but α- and β-RAR transcripts have been detected in a wide variety of cells and tissues. We have measured the level of RAR-α and β transcripts in HUVE cells and our results suggest that these cells contain abundant levels of the β-RAR. Transcripts for this receptor were detected by Northern blot analysis of

total cellular RNA and the identity of these transcripts was confirmed by the isolation and characterization of several β-RAR cDNA clones from a HUVE cell cDNA library. The level of α-RAR transcripts in these cells is much lower, Northern blot analysis of total RNA or poly A-selected RNA failed to detect α-RAR transcripts although screening of the HUVE cell cDNA library did result in the isolation of a single α-RAR cDNA clone.

The abundant presence of retinoic acid receptor transcripts in HUVE cells suggested that they might be retinoic acid responsive. Retinoids have been reported to cause dramatic increases (up to 30-fold) in the expression of transglutaminase activity in cultured cells (22,23) including bovine aortic endothelial cells (4,24). Exposure of HUVE cells to retinoic acid for 24 h did increase the level of transglutaminase activity significantly, but the level of induction (two-fold) was much less than that reported in the bovine aortic cells. This discrepancy may be in part attributable to the relatively high basal level of expression of the enzyme in the human cells but it appears that the maximal level of accumulation of the enzyme in the HUVE cells is less than in bovine aortic cells, where the enzyme accumulates to as much as 2% of total cellular protein (4). It has been reported that cyclic AMP analogues can potentiate the induction of tissue transglutaminase in some cells (25) but dibutyryl cyclic AMP had no effect on the induction of transglutaminase activity in HUVE cells.

Retinoic acid has also been reported to stimulate the expression of the β-RAR (26,27) . In HUVE cells,, however, the β-RAR gene seems to be constitutively expressed, there is a high basal level of expression of receptor transcripts and, under conditions in which it will increase transglutaminase expression, retinoic acid has no effect on β-RAR expression (data not shown). The molecular mechanisms responsible for the constitutive expression of the β-RAR gene in HUVE cells remains to be identified. These cells may be a useful model system for future studies on the factors that regulate retinoic acid receptor gene expression in human cells.

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