Induction of retinoic acid receptor \( \beta \) mediates growth inhibition in retinoid resistant human colon carcinoma cells

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Abstract

Background—The molecular mechanisms underlying the differential sensitivity of human colon carcinoma cells to retinoid mediated growth inhibition are poorly understood.

Aim—to identify the intracellular mechanisms responsible for resistance against retinoid mediated growth inhibition in human colon carcinoma cells.

Methods—Anchorage independent growth of the human colon carcinoma cell lines HT29 and LoVo was determined by a human tumour clonogenic assay. Retinoid receptor expression was evaluated by reverse transcription polymerase chain reaction and northern blotting. Retinoid mediated transactivation was assessed by transient transfection of a pTK: \( \beta \)REx2-luc reporter construct. Retinoid receptor overexpression was achieved by selecting stably transfected cell clones.

Results—Retinoid treatment resulted in profound dose dependent growth inhibition in HT29 cells, while LoVo cells were unaffected. The two cell lines express identical patterns of nuclear retinoid receptor mRNA transcripts. However, on retinoid treatment, retinoic acid receptor \( \beta \) gene expression was upregulated only in retinoid sensitive HT29 cells, but not in retinoid resistant LoVo cells. In accordance, stable overexpression of retinoic acid receptor \( \beta \) but not \( \alpha \) or \( \gamma \) conferred retinoid mediated growth inhibition on LoVo cells.

Conclusion—Induction of retinoic acid receptor \( \beta \) expression is required and sufficient to confer retinoid mediated growth inhibition on human colon carcinoma cells.

The biological effects of retinoids are mainly mediated by two families of nuclear retinoic acid receptors (RARs), each consisting of three receptor subtypes designated \( \alpha \), \( \beta \), and \( \gamma \); the RARs and the retinoid X receptors (RXRs). In addition, each RAR gene generates multiple isoforms by either alternative splicing of exons or differential use of internal promoters. The two retinoid receptor families differ in their ligand and binding affinities: the naturally occurring ligand for RARs in vivo is all-trans-retinoic acid (RA), whereas 9-cis RA preferentially binds to RXRs and RARs. Retinoid receptors are members of the steroid-thyroid hormone receptor superfamily and act as ligand activated transcription factors. RARs and RXRs modulate the expression of their target genes by interacting as either homodimers or heterodimers with cis-acting DNA elements, the so called RAREs (retinoic acid responsive elements) (reviewed in ). Some of the target genes are RARs themselves, in particular the RAR\( \beta \) gene, which contains an RARE (\( \beta \)RARE) in its promoter region. Autoinducion of the RAR\( \beta \) gene has also been suggested to play a central role in mediating the cellular response to retinoic acid. The large spectrum of biological processes affected by retinoic acid as well as the tissue specific restricted expression of RAR/RXR subtypes during embryogenesis and in the adult organism suggests that each RAR and RXR subtype has a unique biological function and thereby determines whether a given cell or tissue is retinoid sensitive or resistant. Therefore detailed knowledge of the cell type specific expression pattern for each receptor subtype may provide a rationale for why certain malignancies or subgroups thereof are retinoid responsive whereas others are not.

In the colon, several experimental studies have looked at the potential antiproliferative effects of retinoids either in a chemopreventive setting of experimental colon carcinogenesis or as antiproliferative treatment of colon carcinoma cells in vivo and in vitro. The results of these studies have been quite contradictory; in the chemopreventive studies on colon carcinogenesis, promising activity as well as lack of biological activity of retinoids in preventing intestinal neoplasia have been reported. Similarly, retinoids have been shown

Keywords: colon cancer; retinoic acid; retinoic acid receptor

Retinoids, the natural and synthetic derivatives of vitamin A, are known to regulate a broad spectrum of biological processes such as development, growth, and differentiation. Their clinical use has so far been focused on the treatment of promyelocytic leukaemia and some epithelial cancers; in addition, retinoids are currently used as chemopreventive and therapeutic agents for a variety of other human premalignancies and tumours.

Abbreviations used in this paper: HTCA, human tumour clonogenic assay; RAR, retinoic acid receptor; RXR, retinoid X receptor; RA, retinoic acid; RT-PCR, reverse transcription polymerase chain reaction; RARE, retinoic acid responsive element.
to inhibit human colon carcinoma cell growth,16–18 or exert no antiproliferative effects.19 Taken together, these data suggest that distinct intracellular mechanisms may determine whether or not a given colon carcinoma cell will respond to the antiproliferative actions of retinoids. We know very little about the mechanisms that determine retinoid sensitivity in human colon cancer cells.

This study was therefore designed as an initial attempt to solve this problem. By using an in vitro system of a retinoid sensitive (HT29) and a retinoid resistant (LoVo) human colon carcinoma cell line, we now provide evidence that retinoid mediated induction of RARβ gene expression is sufficient to confer retinoid sensitivity on a retinoid resistant human colon carcinoma cell line.

Materials and methods

Materials

Human colon carcinoma HT29 and LoVo cells were obtained from the American Type Tissue Culture Collection. Dulbecco’s modified Eagle’s medium was obtained from Gibco (Berlin, Germany), and Dulbecco’s modified Eagle’s medium/Ham’s F12 medium and fetal calf serum from Biochrom (Berlin, Germany). [α-32P]dCTP (6000 Ci/mmol) was from DuPont (Bad Homburg, Germany). Random priming labelling kit and blotting paper were purchased from Amersham (Braunschweig, Germany); RNA molecular size markers, random hexamer primers, and Moloney murine leukaemia virus reverse transcriptase were from BRL (Bethesda, Maryland, USA); restriction enzymes were from Boehringer Mannheim (Mannheim, Germany); Thermus aquaticus (Taq) DNA polymerase was from Promega (Heidelberg, Germany). All retinoids were kindly provided by Hofmann-LaRoche (Basel, Switzerland). All other chemicals were of analytical grade and purchased from Sigma (Deisenhofen, Germany).

Cell culture

Both cell lines were grown as subconfluent monolayer cultures supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml), and streptomycin (100 U/ml). The cells were kept under 95% air and 5% CO2 at 37°C. Medium was changed every two days. Retinoids were prepared under subdued light as stock solutions in dimethyl sulphoxide and aliquots were kept stored at −80°C until used. Retinoids were added to the cultures from stock solutions. Control cells received the same amount of dimethyl sulphoxide, and the final concentration of dimethyl sulphoxide in the medium never exceeded 0.1%. Cell viability was routinely checked by the trypan blue exclusion test and was consistently found to be greater than 95%.

Human Tumour Clonogenic Assay (HTCA)

For evaluation of anchorage independent clonal growth, we used a methylcellulose HTCA exactly as previously described.20 Viability of the cells had to be more than 85% before they were used for an experiment. The final incubation mixture contained the final retinoid concentrations indicated in the Results section, thus tumour cells were exposed to retinoids for the complete assay period. Colony formation was evaluated with an inverted microscope after an incubation period of 10 days at 37°C, pH 7.2, and 5% CO2. Only colonies containing more than 20 cells were counted.

Table 1 Primers used for polymerase chain reaction amplification

<table>
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<th>5′ primer (5′–3′)</th>
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Figure 1 Differential effects of retinoid derivatives on anchorage independent growth. HT29 and LoVo cells were analysed using the human tumour clonogenic assay with the indicated final concentrations of each retinoid analogue (A, all-trans-retinoic acid; B, 13-cis-retinoic acid; C, 9-cis-retinoic acid). After 10 days the number of colonies was evaluated and expressed as percentage of vehicle treated controls. Values are mean (SEM) from three independent experiments, each performed in triplicate. *p<0.05 v controls.
REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS
RNA was isolated exactly as previously described. Reverse transcription was performed using 1 µg total RNA, 100 pM random hexamer primer, 1 mM dithiothreitol, 6 mM Mg2+, 500 µM each dNTP, 20 units RNAsin (Promega, Heidelberg, Germany), and Moloney murine leukaemia virus reverse transcriptase. The reverse transcriptase mixture was used directly as a template for PCR in a 1:20 dilution. Table 1 shows the receptor subtype specific 5' primers and 3' primers used for amplification; they were designed complementary to the human and mouse nucleotide sequences. The expected molecular size of the PCR amplification products was as follows: RARα, 438 bp; RARβ, 435 bp; RARγ, 515 bp; RXRα, 327 bp; RXRβ, 552 bp; RXRγ, 351 bp. The reaction was carried out exactly as previously described.

SOUTHERN BLOT ANALYSIS
After amplification, 10 µl of each RT-PCR mixture was subjected to electrophoresis on a 1.5% agarose gel. Southern blotting was performed exactly as previously described. The following plasmids were used: pSG5:RARα containing a 1.9 kb EcoRI fragment coding for human RARα; pSG5:RARβ containing a 1.4 kb EcoRI-BamHI fragment coding for human RARβ; pSG5:RARγ containing a 1.6 kb EcoRI fragment coding for human RARγ; pSG5:RXRα containing a 1.8 kb EcoRI fragment coding for mouse RXRα; pTL1 containing a 1.35 kb EcoRI-HindIII fragment coding for mouse RXRβ; pSG5:RXRγ containing a 1.5 kb EcoRI fragment coding for mouse RXRγ. Hybridisation was carried out as previously described.

TRANSACTIVATION ASSAYS
The pTK:βREx2-luc reporter construct contains two tandem copies of the RARE of the human RARβ promoter under the control of the herpes virus thymidine kinase promoter linked to the luciferase reporter gene. For transient transfections, colon carcinoma cell lines were transfected with 5 µg of reporter construct plasmid using the Lipofectamine reagent (Gibco-BRL, Eggenstein, Germany) for six hours under serum free conditions. After 12 hours, the indicated concentrations of retinoids or vehicle were added and the mixture incubated for an additional 24 hours. Luciferase activity of cell lysates was then determined using a luciferase assay system (Promega, Heidelberg, Germany). Luciferase activity was expressed as fold induction of vehicle treated control cells.
STABLE TRANSFECTION OF COLON CARCINOMA CELLS

Full length cDNAs of human RARα, β, and γ were isolated from the host vector pSG5 and subcloned in the linearised HindIII site of pRc/CMV vector, containing a neomycin resistance gene. This yielded the RAR cDNAs preceded by the CMV promoter and followed by the human growth hormone termination and polyadenylation signal. The constructs were confirmed by restriction analysis. HT29 and LoVo carcinoma cell lines (about 4 × 10^6 cells per 100 mm dish) were transfected with 5 µg of each plasmid using the Lipofectamine reagent following the instructions supplied by the manufacturer. Control cells were transfected with vector alone (mock transfected) not containing RAR specific nucleotide sequences. At 24 hours after transfection, cells were diluted 1:10 and plated in medium containing 1 mg/ml G418. Resistant cell clones appeared after about 21 days and were picked for expansion at 35 days. The RAR and mock transfected cell clones were kept under selection pressure for all the experiments.

STATISTICAL ANALYSIS

Results were evaluated statistically by analysis of variance followed by a multiple contrast test. p<0.05 was considered to be significant.

Results

DIFFERENTIAL EFFECTS OF RETINOIDS ON HUMAN COLON CARCINOMA CELL GROWTH

We initially investigated the antiproliferative effects of the major naturally occurring retinoid derivatives on anchorage independent growth in HT29 and LoVo cells. Using HTCA, we observed profound dose-dependent growth inhibition in response to all-trans RA in HT29 cells, whereas 9-cis RA treatment resulted in significant growth inhibition only at a maximal concentration of 1 µM (fig 1). In contrast, none of the retinoids tested had any significant growth inhibitory effect on LoVo cells over the concentration range 100 pM to 1 µM (fig 1), suggesting that this cell line is completely retinoid resistant in terms of anchorage independent growth regulation. This in vitro model of a retinoid sensitive and retinoid resistant colon carcinoma cell line should therefore be suitable for studying the intracellular mechanisms that determine sensitivity for retinoid mediated growth inhibition.

IDENTICAL EXPRESSION PATTERN OF NUCLEAR RETINOID RECEPTORS

Different expression patterns of various nuclear receptor subtypes may account for the differential retinoid mediated growth regulation in HT29 and LoVo cells. To investigate this hypothesis, we performed RT-PCR using receptor subtype specific primers and confirmed the specificity of the amplification product by subsequent Southern blotting using the respective receptor subtype cDNA. To exclude amplification of genomic DNA, an
Figure 5 Overexpression of retinoic acid receptor (RAR) restores retinoid sensitivity in LoVo cells. After stable transfection with the RARβ cDNA, overexpression of RARβ mRNA was confirmed by northern blotting (A). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. Transfected (pCMV:RARβ9, pCMV:RARβ12, pCMV:RARβ14), mock transfected (pCMV), and wild type (WT) LoVo cells were then analysed by human tumour clonogenic assay using the indicated concentrations of all-trans-retinoic acid. (B) After 10 days the number of colonies was evaluated and expressed as a percentage of vehicle treated control. Results shown are mean (SEM) from three independent experiments, each performed in triplicate. *p<0.05 v controls.

internal control without prior reverse transcription was performed and consistently found to be negative. To our surprise, however, we observed that the two cell lines expressed identical patterns of retinoid receptors: mRNA transcripts for RARα, β, and γ as well as RXRα and β could be shown repeatedly in both cell lines (fig 2A,B). Furthermore, RXRα and β were also present in both cell lines, whereas mRNA transcripts for RXRγ could not be detected in either cell line (fig 2B). Therefore, differential expression of retinoid receptor subtypes cannot be responsible for the observed differences in retinoid sensitivity between HT29 and LoVo cells.

LACK OF RETINOID MEDIATED RARβ INDUCTION IN LOWO CELLS

Retinoid mediated autoinduction of RARβ gene expression has been suggested to play a crucial role in mediating the biological effects of retinoids. We therefore investigated the effects of all-trans RA on RAR subtype gene expression in both cell lines using northern blot analysis. Incubation with all-trans RA for 24 hours resulted in a selective pronounced increase in RARβ mRNA concentrations in HT29 cells while RARα and γ mRNA levels remained unchanged (fig 3). In contrast, incubation with all-trans RA had no effect on RARβ mRNA concentrations in the retinoid resistant LoVo cell line (fig 3). To confirm further the suspected defect in retinoid mediated autoinduction of the RARβ gene in LoVo cells, we performed transactivation assays using the pTK::βRARE2-luc reporter construct transiently transfected into both cell lines (fig 4). All-trans RA treatment of HT29 cells resulted in a pronounced dose dependent transactivation of the reporter construct, with an approximately 100-fold transcriptional increase mediated by the RARE of the human RARβ gene. In contrast, all-trans RA treatment of LoVo cells did not result in a significant transactivation of the reporter construct, further corroborating the observation that the pathway of retinoid mediated autoinduction of the RARβ gene is deficient in retinoid resistant LoVo cells (fig 4).

OVEREXPRESSION OF RARβ RESTORES RETINOID SENSITIVITY IN LOWO CELLS

We next investigated whether the lack of retinoid mediated RARβ induction is indeed responsible for retinoid resistance in LoVo cells. We therefore stably transfected the RARβ cDNA in LoVo cells and examined the consequences of RARβ overexpression using HTCA. Three independent cell clones (pCMV:RARβ9, 12, and 14) with significant RARβ mRNA overexpression compared with mock transfected controls or wild type parental cells were obtained, as evidenced by northern blotting (fig 5). Overexpression of RARβ did not result in changes in RARα or γ mRNA expression in the transfected cell clones. When these RARβ overexpressing LoVo cell clones were incubated with all-trans RA in HTCA, we observed significant dose dependent growth inhibition of all three cell clones, whereas wild type and mock transfected controls were unaffected (fig 5). To confirm the specificity of the effect mediated by RARβ overexpression, we also overexpressed RARα and γ in LoVo cells. We obtained two stably RARα and one RARγ overexpressing cell clones, as evidenced by northern blotting (fig 6). However, when these stably transfected cell lines were analysed by HTCA, no significant retinoid mediated growth inhibition was observed compared with wild type or mock transfected controls (fig 6).
Anchorage independent growth inhibition was analysed by an HTCA, which has been shown to reflect more reliable growth regulatory phenomena in vitro with tumour growth regulation in vivo.\textsuperscript{23} We tested three therapeutically relevant retinoid derivatives for their ability to inhibit anchorage independent growth in both human colon carcinoma cell lines. All-trans and 13-cis RA treatment resulted in profound dose dependent growth inhibition at therapeutically achievable concentrations\textsuperscript{24} in HT29 cells, whereas the RXR family, which prefers the ligand 9-cis RA, was mainly ineffective. In contrast, even at maximal concentrations, none of the retinoids tested had antiproliferative effects on LoVo cells, suggesting that this cell line is indeed resistant to retinoid mediated growth inhibition.

Assuming that each retinoid receptor subtype exerts a unique biological function, as supported by several studies,\textsuperscript{27–31} it is conceivable that a different retinoid receptor expression pattern in LoVo cells accounts for the lack of biological effects in this cell line. However, RT-PCR analysis using retinoid receptor subtype specific primers showed that the two cell lines express identical repertoires of RARs (\(\alpha\), \(\beta\), \(\gamma\)) and RXRs (\(\alpha\) and \(\beta\)), and RAR\(\gamma\) was not expressed in either cell line. Therefore it appears unlikely that lack of expression of certain retinoid receptor subtypes is responsible for retinoid resistance in LoVo cells.

Recent studies suggested that retinoid mediated autoinduction of the endogenous RAR\(\beta\) gene may play a crucial role in mediating growth inhibition in various cell types.\textsuperscript{32–34} In fact, when retinoid sensitive HT29 cells were incubated with all-trans RA for 24 hours, selective autoinduction of RAR\(\beta\) gene expression was observed, whereas RAR\(\alpha\) and \(\gamma\) mRNA concentrations remained unchanged. In contrast, no autoinduction of RAR\(\beta\) gene expression was observed under identical experimental conditions in the retinoid resistant LoVo cell line. These data were further substantiated by transactivation assays using a human \(\beta\)RARE-luciferase reporter construct. Again, treatment with all-trans RA resulted in potent transcriptional activation (nearly 100-fold) of the reporter construct in HT29 cells, whereas no significant transactivation was observed in LoVo cells. The reasons for the lack of RAR\(\beta\) autoinduction in retinoid resistant LoVo cells are not known. A similar observation has been made in several pituitary cell lines without evidence of mutations in the RAR\(\beta\) promoter,\textsuperscript{34} suggesting either a general defect in the transcriptional machinery\textsuperscript{12, 31} or different expression of cell type specific factors required for retinoid responsiveness. In this context it has recently been shown that E1A-like proteins act as coactivators of retinoid dependent transcriptional activation of the RAR\(\beta\) promoter.\textsuperscript{35, 36} Whether lack of expression or malfunction of these proteins is responsible for the failure to autoinduce RAR\(\beta\) in LoVo cells is under investigation.

To determine further the biological significance of RAR\(\beta\) autoinduction by retinoids (or lack thereof), we generated three independent

These data therefore indicate that RAR\(\beta\) overexpression selectively and specifically confers retinoid mediated growth inhibition on retinoid resistant LoVo cells.

**Discussion**

On the basis of their antiproliferative mechanism of action and the low toxicity profile in clinical studies, retinoids represent an attractive therapeutic approach for chemoprevention and treatment of malignancies.\textsuperscript{1–4} Accordingly, a variety of different retinoids were investigated in a series of preclinical models of colon carcinogenesis and cancer. The emerging picture from these studies is that some colonic cancer cells will respond to antiproliferative treatment with retinoids while others are resistant to growth inhibition.\textsuperscript{11–19} Although much has been learnt over the last few years about the underlying molecular mechanisms of retinoid action, there is currently no information available on the differential retinoid sensitivity of colon cancer cells. In this study we therefore established an in vitro system of two human colon cancer cell lines (HT29 and LoVo) as a model for further investigation of differential response to retinoid mediated growth inhibition.
LoVo cell clones that stably overexpress RARβ mRNA. When these cell clones were analysed by HTCA, significant dose dependent growth inhibition by all-trans RA was observed in contrast to the wild type parental or mock transfected control cells which were not responsive to retinoid. To substantiate further the specificity of RARβ overexpression in mediating retinoid responsiveness, we also generated LoVo cell clones overexpressing RARαs and RARγ. However, although significant overexpression of both receptor subtypes could be shown by northern blotting, neither RARα nor RARγ conferred retinoid responsiveness on LoVo cells in HTCAs. These data therefore indicate that induction of RARγ gene expression is required, and is sufficient, to confer retinoid mediated growth inhibition on otherwise retinoid resistant LoVo colon carcinoma cells. In addition to this study, it has recently been shown that RARα confers retinoid sensitivity on human breast cancer cells,12 RARγ mediates growth inhibition in teratocarcinomas and neuroblastoma cells,17 21 and RARβ determines retinoic acid responsive gene regulation in embryonal cells.20 Taken together, these observations suggest that the biological function of each retinoid receptor subtype depends on the tissue specific context and hence to be determined individually for each cell type.

In summary, our study provides for the first time an experimental model that may explain the differential responsiveness to retinoid mediated growth inhibition previously observed in a variety of preclinical models of colon carcinogenesis and cancer. The ability of a given colon carcinoma tissue to autinduce RARβ gene expression on retinoid treatment may be exploited as a predictive variable to evaluate an antiproliferative response to retinoid treatment.

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