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The additive antiproliferative effect of all-*trans* retinoic acid and interferon-α2a on human cervical carcinoma cell lines is not associated with increased expression of retinoid receptors

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The ability of all-*trans* retinoic acid (atRA), interferon-α2a (IFN-α2a) or a combination thereof to modulate the growth of three human cervical carcinoma cell lines (ME180, MS751 and CaSki) and the relationship between responsiveness and the expression of cytosolic retinoid-binding proteins (CRBP and CRABP II), nuclear RA receptors (RAR-α, -β and -γ) and retinoid X receptor (RXRα) were investigated. atRA induced an antiproliferative effect on two of the cell lines (ME180 > MS751), whereas CaSki was much less responsive. An additive growth inhibition on the latter two cell lines was achieved with the combined treatment of atRA and IFN-α2a. Receptor expression appeared to be unrelated to growth inhibition in these cell lines in so far as atRA exerted minimal effect on the growth of CaSki, although these cells expressed four of these nuclear receptors. However, mRNA for CRABP II was not demonstrable in CaSki, in contrast to the other two atRA responsive cell lines, as evaluated with RT-PCR and ethidium bromide staining. Treatment with atRA or IFN-α2a did not induce any change in mRNA for the nuclear retinoid receptors or cellular retinoid binding proteins after 3 or 6 days of treatment.

Key words: All-*trans* retinoic acid, cervix uteri, growth inhibition, interferon-α, squamous cell cancer.

Introduction

Retinoids and interferons are biological response modifiers which regulate many cellular functions. The retinoids exert their multiple biological effects through all-*trans* and 9-*cis* retinoic acid and two families of nuclear receptors (RAR-α, -β and -γ, and RxR-α, -β and -γ), which regulate gene expression[1,2] and play a role in the effects of retinoids on cellular differentiation and proliferation.[3–5] Cytoplasmic retinol-binding protein, CRBP, and retinoic acid-binding proteins, CRABP I and II, are probably of importance for the intracellular transport and metabolism of retinoids.[6]

Interferon-α (IFN-α) is comprised of a group of proteins produced by the body in response to viral infections. Besides their antiviral activity, IFN-α regulate normal and malignant cell growth and differentiation.[7,8] Among other things, IFN-α decrease the expression of several oncogenes, and modulate growth factors, cell cycle regulators and cell surface antigens.[9,10] Preclinical studies indicate that combinations of retinoids and various interferons have additive or synergistic anti-proliferative, differentiating and anti-angiogenic activity in human hematologic and solid tumor systems.[11–18] The combined treatment with 13-*cis*-retinoic acid and IFN-α2a has shown promising results in recent clinical trials on previously untreated squamous carcinoma of the cervix and advanced pretreated squamous carcinoma of the skin,[19–21] whereas no effect was achieved with this combination on recurrent heavily pretreated cervical carcinomas.[22]

The molecular mechanisms behind the interaction between retinoids and IFN are still unknown. The purpose of this study was to evaluate the effect of all-*trans* retinoic acid (atRA) and IFN-α2a on the growth of human cervical carcinoma cell lines, to study whether they interact at the level of nuclear receptors for retinoic acid (RAR-α, -β and -γ, and RxR-α) or cellular retinoid-binding proteins (CRBP and CRABP II).

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Materials and methods

Chemicals

atRA, a generous gift from Roche (Stockholm, Sweden), was dissolved in dimethylsulfoxide (DMSO) to a concentration of 10 mM and this stock solution was stored in the dark at −70°C. The retinoid was diluted with culture medium to obtain the final concentration in the range of 10^{-9} to 10^{-6} M. Human recombinant IFN-α2a (specific activity of 2×10^8 IU/mg protein), a kind gift from Roche, was dissolved in culture medium containing DMSO 0.01% (v/v). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and dimethylsulphoxide (DMSO) were purchased from Sigma (Stockholm, Sweden).

Tumor cell lines

Three human cervical carcinoma cell lines, MS 751, ME 180 and CaSki, were obtained from Dr Björk-Eriksson (Sahlgrenska Hospital, Gothenburg, Sweden). The properties of these cell lines are outlined in the ATCC catalogue (Rockville, MD). The cells were propagated in DMEM (Dulbecco), supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 0.06 mg/ml benzyle penicillin and 0.1 mg/ml streptomycin. The cells were routinely cultured as adherent monolayers in 25 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 in air. The cell lines were not infected with mycoplasma, as tested with DNA staining with Hoechst 33258.

Measurement of cell proliferation

Assessment of viable cell number was evaluated with the MTT dye reduction assay.[23] Cells were harvested from subconfluent cultures by treatment with 0.25% (w/w) trypsin and plated into flat-bottom 96-well microtiter plates at an initial mean density of $0.5-1 \times 10^3$ cells/well in 100 μl medium. Analyses were made to establish the relationship between cell number and absorbance, measuring the amount of formazan produced. This relationship was linear over the range of $1-50 \times 10^3$ cells/well. When $0.5-1 \times 10^3$ cells/well were plated, the number of cells/well after 6 days of incubation was within this range. After a 24 h preculture period to ensure attachment, 100 μl fresh medium with the appropriate dilutions of retinoid or IFN was added. At 72 h intervals thereafter, the medium was replaced with fresh medium containing the indicated concentration of atRA, IFN-α2a, a combination thereof or control medium containing 0.01% DMSO. After 6 days of incubation, 10 μl of MTT dye at 5 mg/ml in PBS was added to each well. After an additional 4 h incubation at 37°C, the supernatant was removed and 150 μl 100% DMSO added to each well to solubilize the formazan precipitate. Within 30 min, the plates were read at 570 nm on a Multiscan plate reader. Growth inhibition was calculated according to the formula: GI(%) = (1 − OD experimental/OD control) × 100; where OD = optical density (absorbance level in nm). The IC_{50} was determined by interpolation from dose–response curves. All experiments for RA effects were performed under light protection.

RNA purification and RT-PCR analysis

Cells were seeded in plastic tissue culture flask at 6×10^4 cells per flask and cultivated as described. After 24 h, and at 72 h intervals thereafter, the medium was replaced with fresh medium containing atRA (10^{-6} M), IFN-α2a (500 U/ml), a combination thereof or control medium containing 0.01% DMSO. After 3 and 6 days of incubation, the medium was removed and the cells were dissolved in 4 mol/l guanidine isothiocyanate in 25 mmol/l citrate buffer pH 7 and total RNA extracted as described.[24] The concentration of RNA was judged from the absorbance at 260 nm and RNA purity from the absorbance ratio A260/A280. The RNA preparations generally had absorbance ratios of more than 1.4–1.5. The mRNA of the retinoid-binding proteins, cellular retinoic acid-binding protein type II and cellular retinol-binding protein, CRABP II[25] and CRBP,[26] retinoid X receptor alpha, RXR-α,[27] the retinoid receptors, RAR-α, -β and -γ,[28–30] and β-actin (internal standard), were quantitated by RT-PCR with kits from Perkin Elmer and Promega, according to the instructions in the kit. The primers used for the RT-PCR are shown in Table 1. We tried the annealing PCR temperature indicated by the theoretical $T_m - 5$°C and at least two different temperatures up to ±1°C from the temperature indicated by the theoretical T_m, before a negative result was accepted. In our experience the method used is somewhat less sensitive than Northern blotting but gives cleaner results and is much nicer to work with.[31]

The resulting amplified fragments were visualized by agarose gel electrophoresis and ethidium bromide

Table 1. Oligonucleotides for reverse trancriptase-polymerase chain reaction

Protein	Sequence	Location in protein	Size of PCR product (bp)	Reference
CRBP	5′-GTCGACTTCACTGGGTAC-3′ 5′-TCACTGCACCTTCTTGAATA-3′	Val2–Tyr7 Val129–Stop135	402	26
CRABP II	5′-ATGCCCAACTTCTCTGGC-3′ 5′-TCACTCCTCGGACGTAGAC-3′	Met1–Gly6 Val134–Stop139	417	25
RXR-α	5′-TGGCAAGGACCGGAACGAGAATG-3′ 5′-GCGGCGCCTCCAGCATCTCCATA-3′	Gly212–Glu219 Leu451–His459	743	27
RAR-α	5′-CATTGAGAAGGTGCGTAAAGCG-3′ 5′-GGCCGGCTGCTTCTGTTGG-3′	Leu158–Ala164 Asn423–Ala428	794	28
RAR-β	5′-CAAATTACCCTGCTGAAGG-3′ 5′-CACGAGTGGTGACTGACT-3′	Gln250–Lys255 Ser442–Val447	591	29
RAR-γ	5′-GACGGGCTGACCCTGAA-3′ 5′-TCAGGCTGGGGACTTCA-3′	Asp290–Leu295 Glu450–Stop455	492	30
β-Actin	5′-ATGGATGATGATATCGCCG-3′ 5′-ATGAGGTAGTCAGTCAGGT-3′	base 41–60 base 591–610	568	

staining. The ethidium bromide-stained bands were scanned with a BioRad model GS-670 scanner and evaluated with a BioRad program package, Molecular analyst. The same area was measured in sets of four to six samples to ensure comparability between samples. The area so obtained for the RARs and the RXRs and the retinoid-binding proteins was divided by the area measured for β-actin (internal standard), to create a measure where differences in amount of starting RNA applied were eliminated. mRNA for actin was chosed as it is not regulated by IFNs or retinoids. If the message was demonstrated we did not consider differences up to one magnitude as real differences, due to the great variability of the method,[31] but also knowing that induction usually produced differences amounting to several orders of magnitude. In some cases where no product could be visualized by ethidium bromide staining, and, therefore, no band could be detected, the gels were blotted on to Hybond N membranes and hybridized to probes ^{32}P labeled by random priming with [^{32}P]CTP (Amersham). The templates used were full-length RAR-α, -β and -γ cDNA (obtained from Dr A Dejean, Paris, and Dr P Chambon, Strasbourg, France), and full-length CRBP cDNA (from Dr U Eriksson, Stockholm, Sweden). CRABP II exon 2 was amplified from genomic DNA isolated from human peripheral blood using 20-mer oligonuclotides corresponding to the sequence between Val25 and Met83 and the resulting fragment cloned. The cDNA for β-actin (2000 bp) was obtained from Dr C Holm, Department of Biochemistry, University of Lund. After prehybridization for 4 h at 42°C, the probes were hybridized to the transferred fragments at 42°C overnight. The papers were then washed with 2 × SSC for 5 min at 20–23°C for 45 min in 0.5 × SSC 0.1% (w/v) SDS at 60°C and for 30 min in the same buffer at 63°C and then put on XAR 5 film at −70°C for variable lengths of time.

Results

Antiproliferative effects of atRA and IFN-α2a

atRA (10^{-9} to 10^{-6} M) alone caused a variable dose-dependent growth inhibitory effect after a 6 day treatment on the three cervical carcinoma cell lines studied, as shown in Figure 1. ME180 was most sensitive to the antiproliferative activity of atRA with IC_{50} of 8×10^{-8} M (Figure 1a). MS751 was less sensitive with a maximal inhibition of 30% at 10^{-6} M (Figure 1b), whereas CaSki showed no significant response (Figure 1c).

All three cell lines were very moderately to minimally responsive to IFN-α2a treatment alone. IFN-α2a at a clinically achievable concentration of 100 U/ml resulted in a growth inhibition of less than 15% in all three cell lines (Figure 1a–c).

An additive inhibitory effect was achieved with combined treatment with atRA and IFN-α2a on ME180 and MS751 as shown in Figure 1(a–b). With suboptimal concentrations of the agents not more than additive effects could be demonstrated. CaSki cells, which were insensitive to both atRA and IFN-α2a, did not exhibit sensitivity to the combination of the two substances either.

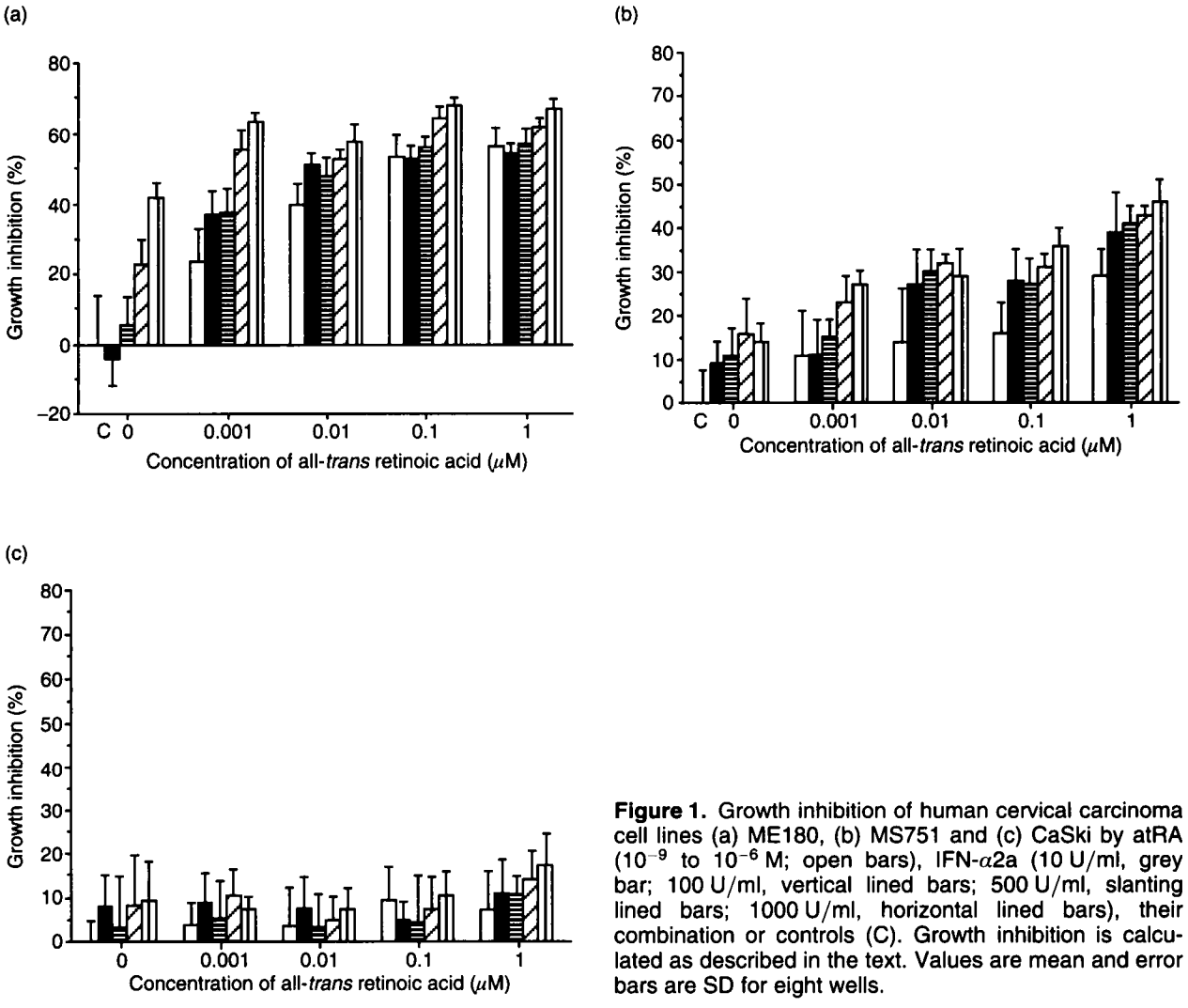

Figure 1. Growth inhibition of human cervical carcinoma cell lines (a) ME180, (b) MS751 and (c) CaSki by atRA (10^{-9} to 10^{-6} M; open bars), IFN-α2a (10 U/ml, grey bar; 100 U/ml, vertical lined bars; 500 U/ml, slanting lined bars; 1000 U/ml, horizontal lined bars), their combination or controls (C). Growth inhibition is calculated as described in the text. Values are mean and error bars are SD for eight wells.

Expression of retinoid receptors and retinoid-binding proteins

The expression of nuclear retinoic acid receptors (RXR-α and the RARs), cytoplasmic retinoic acid-binding protein (CRABP II) and cellular retinol-binding protein (CRBP) in the cell lines was analyzed by RT-PCR, the electrophoretic gels were stained with ethidium bromide and the area of the resulting bands determined with a scanning densitometer. In some cases the expression was determined with a Northern blot.

RXR-α, which was the major retinoid receptor that could be measured, was demonstrable in all cell lines (Table 2). RAR-α and RAR-γ was also expressed in ME, MS and CaSki at approximately similar levels in the cell lines. RAR-β mRNA was demonstrable at low and variable levels by RT-PCR in all cell lines, in some cases below the sensitivity of ethidium bromide staining and densitometric scanning, but demonstrable by hybridization with radiolabeled probes (Northern blot).

Treatment with atRA (10^{-6} M) did not induce any change in mRNA for the nuclear retinoid receptors after 3 or 6 days of treatment, nor did IFN-α2a or the combined treatment induce any changes in expression of these receptors (Table 2).

The cell lines ME and MS both expressed high levels of CRABP II, whereas mRNA for CRABP II was not demonstrable in CaSki. CRBP was expressed by all cell lines. In none of the cell lines was there any marked induction of mRNA for CRABP II or CRBP with retinoid (or IFN-α2a) treatment.

Thus, as evaluated with RT-PCR and ethidium bromide staining of the products, lower or higher levels of gene expression of the retinoid receptors

Table 2. Effect of the various treatments on the expression of retinoid receptors and retinoid-binding proteins relative to β-actin after 3 days of culture

	ME				MS				CaSki			
	Controls	atRA	IFN-α2a	atRA + IFNα2a	Controls	atRA	IFN-α2a	atRA + IFNα2a	Controls	atRA	IFN-α2a	atRA + IFNα2a
CRABP II	1.38	1.08	0.98	1.11	0.85	0.93	1.01	1.09	ND	ND	ND	ND
CRBP	0.32	0.34	0.22	0.38	0.32	0.37	0.13	0.11	0.20	0.88	0.26	0.70
RXR-α	1.15	0.88	1.05	0.95	0.91	0.81	0.71	1.27	0.08	0.11	0.03	0.26
RAR-α	0.04	0.13	0.34	0.03	0.61	0.55	0.05	0.11	0.23	0.34	0.33	0.51
RAR-β	0.34	0.11	0.09	ND	0.31	Nm	Nm	Nm	0.26	0.63	0.44	0.53
RAR-γ	0.44	0.40	0.30	0.41	0.56	0.45	0.37	0.56	0.34	0.60	0.12	0.22

Results of scanning of the surface area of ethidium bromide-stained RT-PCR (using the primers of Table 1) of retinoid receptors and retinoid-binding proteins. Results are expressed as ratios between the surface area obtained for each retinoid receptor or retinoid-binding protein and β-actin.
ND, not detected by ethidium bromide staining, Nm, not measured.

or the retinoid binding proteins paralleling the growth inhibition could not be demonstrated in any of the cell lines, possibly with the exception of CRABP II in CaSki.

Discussion

In the study presented, we examined the effect of atRA, IFN-α2a or combined treatment on the growth of three cervical carcinoma cell lines, and the relationship between that and the expression of its nuclear receptors and retinoid-binding proteins. A dose-dependent anti-proliferative effect of atRA and IFN-α2a and an additively enhanced growth inhibiting effect of their combination was achieved on ME180 and MS751 in accordance with previous studies.[15–17] CaSki showed poor responsiveness to atRA, but contrary to that found by Agarwal *et al.*[14], atRA did not promote growth, although the growth conditions in this study were not equal to theirs. Most studies, consistent with this study, have found more additive than synergistic growth inhibition with the combined treatment of atRA and IFN-α2a on squamous cell carcinoma lines, of cervical as well as of oral origin.[13,17,32] It has also been shown that more than additive inhibition of proliferation was obtained by combining RAR- and RXR-selective ligands, and that further proliferation inhibition could be accomplished by adding IFN-α.[16]

It is noteworthy that the cell lines sensitive to atRA, i.e. ME180 and MS751, express similar levels of nuclear retinoid receptors to the retinoid-insensitive cell line CsSki, as shown previously.[16,17] The sensitivity to retinoids calls for an intact retinoid signal system. However, an intact retinoid signaling system is apparently not enough as insensitive cell lines, like CaSki, express the RARs and RXRα at similar levels to retinoid-sensitive cell lines. CRABP II, on the other hand, was not demonstrable by ethidium bromide staining in CaSki cells, which means that if expressed at all, it is expressed at a lower level than in ME and MS cells. The difference between the cells with regard to the response to retinoids must lie at some point either in conjunction with the uptake or metabolism of retinoic acid in the cells or distal to the expression of RXR-α and the RARs. CRABPs have been suggested to be proteins linked to RA cellular metabolism and in controlling the level of RA available for interaction with the nuclear receptors.[6,33] It is intersting that the ability of retinoid metabolism seemed to be correlated with retinoid sensitivity in a variety of epithelial and epithelial cancer cell lines.[34] On the other hand, it has been proposed that CRABP may play a negative role in retinoid-mediated signal transduction by sequestering RA in the cytoplasm and enhance its catabolism with the result that a lower RA concentration reaches the cell nucleus.[33] One of four head and neck squamous cell carcinoma cell lines responsive to RA did not express CRABP II.[35] The results discussed above concerning the relationship between responsiveness and expression of receptors and retinoid-binding proteins should be taken with some caution as the latter is based primarily on analysis of mRNA, while their post-transcriptional regulation and mutations are not considered.

A decreased expression of RAR-β is suggested to be associated with the development of head and neck cancer and lung cancer.[36,37] Most of the immortalized human cervical carcinoma cell lines, in

this study as in a previous study,[16] express RAR-β, although at different levels and the expression of RAR-β was not correlated to the response to retinoids. No significant induction of RAR-β was seen either in ME, MS or Caski upon retinoid treatment despite the presence of RAREs in the genes of RAR-β and CRABP II.[17]

The molecular mechanism of the interaction of retinoids and interferons is still unknown. In ME180 the combined treatment of atRA plus IFN-α2a induces an increase in the expression of 2-5A synthetase and transcriptional factor IRF-1 genes.[15] IFN-γ was shown to augment the expression of RAR-γ in breast cancer cells,[38] but similar changes in RARs with IFN-α2a were not found in the studied cervical carcinoma lines.

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