0022-5347/05/1731-0247/0
THE JOURNAL OF UROLOGY®

Vol. 173, 247–251, January 2005
Printed in U.S.A.
DOI: 10.1097/01.ju.0000141584.50650.26

EFFECT OF RETINOIC ACID AND INTERFERON α-2a ON TRANSITIONAL CELL CARCINOMA OF BLADDER

CHANGPING ZOU,* SANJAY RAMAKUMAR, LIXIN QIAN, CHANGCHUN ZOU, RONGYU ZANG, JIAN WANG, H. BARTON GROSSMAN,† REUBEN LOTAN AND MONICA LIEBERT‡

From the Department of Obstetrics/Gynecology and Urology, University of Arizona (CZ, SR, LQ, CZ, RZ, JW), Tucson, Arizona, M. D. Anderson Cancer Center (HBG, RL), Houston, Texas, and American Urological Association (ML), Baltimore, Maryland

ABSTRACT

Purpose: Retinoids modulate the growth and differentiation of normal and malignant epithelial cells in vitro and in vivo. Retinoids and their analogues have been used in animal models and clinical trials of chemoprevention and superficial bladder cancer treatment. Interferons are cytokines that have antiviral, antiproliferative and immunomodulatory function. They are used in many clinical trials for the treatment of different cancers. To identify new effective agents and develop novel approaches for the chemoprevention and treatment of superficial bladder cancer we investigated the effects of a combination of retinoids and interferon α-2a (IFN) on growth and apoptosis in bladder cancer cell lines.

Materials and Methods: The 4 bladder cancer cell lines UM-UC-6, UM-UC-9, UM-UC-10 and UM-UC-13 were treated with 2 retinoids, namely all-trans-retinoic acid (ATRA) and 9-cis retinoic acid (9cRA), as well as with IFN or with combinations of retinoids and IFN. The ability of these agents used alone and in combination to inhibit growth, induce apoptosis and modulate gene expression was investigated. The effects of retinoids on an INF related gene were also examined.

Results: Most bladder cancer cell lines were resistant to growth inhibition and apoptosis induction by ATRA and 9cRA, even at a high concentration. The effects of these retinoids on cell growth and apoptosis were enhanced by IFN. The combination of ATRA and IFN induced retinoic acid receptor β, and signal transducer and activator of transcription 1 expression in 3 bladder cancer cell lines, as detected by reverse transcriptase-polymerase chain reaction and Western blot analysis. Retinoids increased IFN-related gene expression detected by microarray analysis and real-time reverse transcriptase-polymerase chain reaction.

Conclusions: The results demonstrate that IFN acts synergistically with ATRA and 9cRA in the growth and apoptosis of bladder cancer cells in vitro and suggest that this combination has a potential for the treatment of transitional cell carcinoma of the bladder.

KEY WORDS: bladder; carcinoma, transitional cell; retinoids; interferons; apoptosis

More than 80% of patients with a first bladder cancer have tumors that are superficial. These types of tumors have low invasive potential but 50% to 80% of these patients experience recurrence within 12 months after initial resection.[1,2] Primary chemoprevention of bladder cancer and secondary chemoprevention of recurrent bladder cancer are goals for physicians and researchers that have not yet been achieved.

Retinoids regulate the growth and differentiation of normal and malignant epithelial cells in vitro and in vivo, and they have been intensively investigated as chemopreventive agents for the treatment and prevention of various cancers.[3,4] Retinoids have been shown to inhibit bladder carcinogenesis in animal models, although data in man have been less convincing.[5,6] Retinoid receptors were thought to have an important function in mediating the effects of retinoids.[7,8] Two types of receptors have been identified, namely retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs bind to all-trans-retinoic acid (ATRA), while 9-cis-retinoic acid (9cRA) (Sigma Chemical Co., St. Louis, Missouri), a natural retinoic acid isomer, binds to RARs and RXRs.[7,8] Because each subtype of RAR or RXR shows distinct patterns of expression and different distributions, each one is thought to regulate the expression of distinct genes.[7,8]

Interferons (IFNs) are cytokines that have antiviral, antiproliferative and immunomodulatory functions.[9] There are 2 types of IFNs, namely types I (IFN-α and IFN-β) and II (IFN-γ). IFN-α and IFN-γ use different cell surface receptors, and partially different tyrosine kinase, and signal transducer and activator of transcription (STAT) in their signal transduction. The anticarcinogenic effects of IFN are the result of induced changes in cell growth and differentiation caused by changes in the expression of specific genes, such as oncogenes, growth factors and growth factor receptors.[9,10] IFNs exert their effect on gene expression by activating a signal transduction pathway in which STAT protein has an important role. IFN-α and IFN-γ can activate STAT1 protein. IFN regulate factor regulates the expression of IFN and IFN inducible genes that can be activated by IFNs and retinoids.[9,10]

We have previously reported that abnormalities in receptor expression were found in human bladder cancer cell lines.[11] RARβ expression was suppressed in most bladder cancer cell lines.[11] Abnormalities in RARβ expression suggest that this receptor may be involved in bladder cancer. A combination of retinoids and IFN has been used for phase II treatment of invasive cervical cancer and renal cancer.[12,13] Our group as well as others has reported that a combination

Submitted for publication April 21, 2004.
Supported by National Institutes of Health/National Cancer Institute CA75966.
* Correspondence: Department of Obstetrics/Gynecology, College of Medicine, University of Arizona Cancer Center, Tucson, Arizona 85724 (telephone: 520-626-8883; FAX: 520-626-9287).
† Financial interest and/or other relationship with Fujirebio Diagnostics, PhotoCure, Abbott/Vysis, AstraZeneca, Pharmacia and UroCor.
‡ Financial interest and/or other relationship with UroCor and Department of Defense.

of retinoids and IFN had a synergistic effect on growth inhibition of cervical cancer cells.[14] A combination of these 2 agents also had signaling cross-talk in normal and retinoid resistant acute promyelocytic leukemia.[15] We now describe the effects of ATRA (RAR pathway) and 9cRA (RXR pathway) used alone and in combination with IFNα-2a on growth inhibition, apoptosis, and RARβ and STAT1 expression in bladder cancer cell lines.

MATERIALS AND METHODS

Cell lines and reagent. The bladder cancer cell lines UM-UC-6, UM-UC-9, UM-UC-10 and UM-UC-13 were derived from transitional cell carcinomas.[16] Cells were grown in a 1:1 (volume per volume) mixture of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37C in a humidified atmosphere of 95% air and 5% CO_2.

Retinoids used in the study were ATRA and 9cRA. Retinoids were dissolved in dimethyl sulfoxide as stock solutions of 0.1 mM and stored in an atmosphere of N_2 at −80C.

Effects of IFN and ATRA on cell proliferation in monolayer cultures. Cells were plated in 96-well plates at a concentration of 10^4 or 2×10^4 cells per well and grown for 24 hours. Cells were then incubated with 100, 500 and 1,000 U IFNα-2a, and 1 μM concentrations of ATRA and 9cRA. Control cultures contained dimethyl sulfoxide. Growth inhibition was determined using the crystal violet method, as described previously.[11] Fixed and stained cells were read by using a plate reader (BioRad Laboratories, Hercules, California). Growth inhibition was calculated according to the equation, inhibition % = $(1 - Nt/Nc) \times 100$, where Nt represents the number of cells in treated cultures and Nc represents the number of cells in control cultures. All experiments were performed in triplicate and the mean ± SD was calculated.

Analysis of apoptosis induced by IFN and retinoids. The TUNEL assay was performed, as previously described.[11] Following incubation with 1 μM ATRA or 9cRA, or 1,000 U IFNα-2a alone and in combination for 3 days cells were fixed in 1% formaldehyde. Cells were first suspended in 1 ml wash buffer from a flow cytometry kit (Phoenix Flow Systems, San Diego, California). Approximately 10^6 cells were resuspended in 50 μl staining buffer. Cells were stained with 500 μl propidium iodide/ribonuclease A solution and then analyzed by flow cytometry using a FACScan flow cytometer (Epics Profile, Coulter Corp., Hialeah, Florida) with a 15 mW argon laser used for excitation at 488 nm. Fluorescence was measured at 570 nm. Computer analysis of the data provided information on the percent of apoptotic cells.

Analysis of nuclear retinoid receptor β and IFNγ 1 mRNA expression by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Real-time quantitative RT-PCR was performed using a 7700 Sequence Detector (Applied Biosystems, Foster City, California). Specific quantitative assays for RARβ and IFNγ were developed using Primer Express software (Applied Biosystems). Total RNA was extracted from bladder cells by Tri-reagent. Resulting RT-PCR data were analyzed using SDS software (Applied Biosystems). Final data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and they are presented as molecules of transcript/molecules of GADPH × 100 (percent GAPDH). For this type of analysis it is critical that the housekeeping gene does not change across experimental conditions. ΔCt was calculated for each gene by Excel (Microsoft, Redmond, Washington) according to the equation, $\Delta Ct = Ct_{\text{treatment condition}} - Ct_{\text{control condition}}$ 2^(- ΔΔCt) = fold change, where Ct represents the cycle at which the reaction crossed the threshold. If the fold change was greater than 1.0, the gene was up-regulated relative to the control (equal to 1). If the fold change was less than 1.0, the gene was down-regulated relative to the control.

Microarray analysis. UM-UC-6 cells were treated with 1 μM ATRA and synthetic retinoic acid (4HPR) for 3 days. RNA was purified as previous described.[11] RNA samples were assessed for degradation and DNA contamination on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California). Two separate RNAs (control and treated group) were submitted for each microarray chip. Dual label cDNA hybridization chips (10,000 cDNAs per chip) for high density cDNA microarrays were used in the assay to identify transcripts subject to differential expression. An Erie Scientific Lifterslip (Erie Scientific, Portsmouth, New Hampshire) was used for hybridization. Hybridization was performed in a regular hybridization chamber, as recommended by the manufacturer. The recommended washes were followed. Washed chips were read on a Scan Array Lite and the digital image output analyzed using QuantArray software (Perkin-Elmer, Foster City, California). All experiments were performed in triplicate and the mean ± SD was calculated.

Western blot analysis of STAT gene expression. Nuclear and cytoplasmic extracts were prepared from control, and ATRA and IFN treated bladder cancer cells, as previously described.[11] Nuclear or cytoplasmic protein (30 μg per lane) was electrophoresed on 8% polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were incubated with mouse IgG monoclonal antibodies against STAT1 to 3 (Santa Cruz Biotechnology, Santa Cruz, California), washed and incubated with peroxidase conjugated antimouse antibody as a second antibody (Amersham International, Arlington Heights, Illinois). Immunoreactive bands were developed using an enhanced chemiluminescence reagent (Amersham International). The blots were stripped and then re-incubated with mouse anti-β-actin antibody (Sigma Chemical Co., St. Louis, Missouri) to normalize for loading differences.

RESULTS

Growth inhibitory effect of retinoids on human bladder cancer cell lines. The combination of ATRA, 9cRA and IFN as well as each drug used alone was compared for the effect on the growth of 4 human bladder cancer cell lines grown in monolayer cultures. Cells were treated for 5 days at different concentrations of IFN alone and IFN combined with retinoids. Bladder cancer cells were resistant to most retinoids (ATRA and 9cRA), as we have previously found.[11] Dose dependent responses to IFN were observed in UM-UC-6, UM-UC-9 and UM-UC-13 cells (fig. 1). UM-UC-10 cells were resistant to ATRA and IFN (fig. 1). The combination of IFN and ATRA had a greater growth inhibitory effect than any drug used alone (fig. 1). There were no significant changes in morphology when cells were treated with ATRA and IFN alone. Cell number decreased significantly after combination drug treatment (fig. 2).

IFN and retinoid induced apoptosis. We tested the effect of ATRA, 9cis RA or IFN on apoptosis induction. Based on the growth inhibition study 1 μM ATRA and 9cRA, and 1,000 U IFN were used in the experiment. Cells were treated for 3 days and apoptosis was analyzed by the method of terminal deoxynucleotidyl transferase labeling and flow cytometry. Figure 3 shows DNA content and apoptosis induction in the UM-UC-9 bladder cancer cell line. Results demonstrated that ATRA and 9c RA as well as INF alone had little effect on inducing apoptosis (fig. 3). The combination of IFN and retinoids was more potent in inducing apoptosis than either drug used alone (fig. 3). The combination of IFN and 9cRA (48.9%) was a stronger inducer of apoptosis than the combination of IFN and ATRA (22.3%). Cell cycle analysis showed that treatment with IFN and ATRA or 9cRA increased the percent of cells in the G1 phase only slightly compared with control cells, whereas it was significantly increased by the combination of IFN and 9cRA (fig. 3, right).