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Interferon treatment enhances the expression of underphosphorylated (biologically-active) retinoblastoma protein in human papilloma virus-infected cells through the inhibitory TGFβ1/IFNβ cytokine pathway

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Summary

Interferons (IFN) regulate transcription of certain genes playing a role in cell proliferation. Targets of IFN action may include tumor suppressor genes such as the retinoblastoma (RB) gene and cytokines such as transforming growth factor β1 (TGFβ1) and IFNβ which are inhibitors of epithelial cell proliferation. Using reverse transcription followed by PCR amplification, an increase of those growth inhibitory gene mRNA levels (TGFβ1, IFNβ and RB) were found after interferon treatment in condylomas harboring non-oncogenic human papilloma virus (HPV 6/11) types, in an oncogenic HPV 16-containing cell line and in a HPV negative, epidermoid carcinoma cell line. In addition, immunodetection by Western blot demonstrated a higher proportion of underphosphorylated (active form) retinoblastoma gene protein (pRB) after IFN treatment due to the decrease in the phosphorylating cdc2 kinase levels. Changes in the phosphorylation pattern of pRB together with the increased expression of those inhibitory genes represent a growth inhibited state in those cells as demonstrated by diminished c-myc expression. Since the extent of c-myc inhibition was significantly lower in the case of oncogenic HPV infection, a role of viral oncoproteins in abrogation of the antiproliferative effect of IFN therapy could be considered. These results demonstrate a new mechanism via which IFNs exert their antiproliferative effect on HPV-infected cells by

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affecting the expression and phosphorylation of the RB tumor suppressor gene, through the inhibitory TGFβ1/IFNβ cytokine pathway.

Interferons; Retinoblastoma protein; Human papilloma virus; Cytokine pathway

Introduction

The interferons (IFNs) are one of the body's natural defensive responses to such foreign components as microbes, tumors and antigens. Treatment of cells with IFNs can lead to establishment of an antiviral state, inhibition of cell growth and induction of differentiation (Baron et al., 1991). These effects involve an action on the expression of growth factors, oncogenes or other growth stimulating agents (Friedman, 1989). Recent advances have led to approval of certain clinical indications for IFNα, such as hairy cell leukemia, condyloma acuminatum, Kaposi's sarcoma, non-A, non-B (type C) viral hepatitis and hepatitis B as well as approval of IFNγ for treatment of chronic granulomatous disease. Condyloma acuminatum – caused by 'low risk' (non-oncogenic) types of human papilloma viruses (HPV 6 or 11) (zur Hausen, 1991), is by far the most common disease for which the effectiveness of IFNs have been demonstrated. In a number of published studies, IFN was effective in eradicating all visible lesions in 60–70% of patients with condyloma acuminatum who previously had recurrences after a variety of non-antiviral therapies (Tyring, 1988). The amount of virus is thought to be sufficiently reduced in successfully treated warts so that the host's immune system may prevent recurrences from HPV lying latently in clinically normal appearing skin.

In our preliminary studies a significant decrease in HPV 6/11 copy number was found in the tumors from patients with condyloma acuminatum after IFN treatment (Arany et al., 1993). In those experiments we also demonstrated that 'low risk' HPV infection downregulates some inhibitory cytokine gene mRNA levels, like endogeneous TGFβ1 and IFNβ expression, but upregulates cdc2 kinase mRNA levels in condylomas confering growth advantage to them. Since these genes are known to play important roles in skin keratinocyte proliferation (Moses et al., 1990), but little is known about their role in IFN therapy, we determined mRNA and protein levels expressed by those genes in different epithelial cells infected with different HPV types, in vivo and in vitro, before and after IFN treatment.

Materials and Methods

After signing an informed consent, patients were given local anesthesia in the area of their condyloma using 1% lidocaine; one wart was then surgically

excised and immediately frozen at −70°C until processed. Patients then were treated with 1.5×10^6 units of IFNα2a (Roferon A, Hoffmann La Roche, Nutley, NJ) plus 1.5×10^6 units of IFNγ (Actimmune, Genentech, South San Francisco, CA). These injections were given subcutaneously, bilaterally in the crual folds three times per week for 6 weeks. Post-treatment biopsies of condyloma or clinically normal skin at the site of former condyloma were taken 6 to 12 weeks following the last IFN treatment. These specimens were also immediately frozen at −70°C until processed.

Cells from the A431 epidermoid carcinoma line (ATCC CRL 1555) or from the CaSki line (ATCC CRL 1550) were grown in media suggested by the ATCC (DMEM with 5 g/l glucose with 10% FBS or RPMI 1640 with 10% FBS, respectively) until the optimal cell concentration was achieved for observance of any cell growth inhibition/cell death. The media was then decanted and replaced by the same media with or without 500 U/ml IFNα2a (Roferon A, Hoffmann La Roche, Nutley, NJ) plus 500 U/ml IFNγ (Actimmune, South San Francisco, CA). At the end of 48 h incubation, the cell viability was determined and those cells were used for further studies.

Total RNAs and cellular proteins were simultaneously isolated from frozen biopsy samples or cell pellets using Tri-Reagent™ (Molecular Research Center, Cincinnati, OH).

To simultaneously determine different gene transcripts, 1 μg of RNA was subjected to cDNA synthesis at 42°C for 1 h using Superscript RNAse H^- reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and random hexamer priming (Promega, Madison, WI) for the first strand synthesis. This cDNA mixture was aliquoted and PCR amplifications were done under the same conditions using different gene specific primer pairs, and co-amplified with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as internal control. Primers for G3PDH, IFNβ and TGFβ1 were obtained from Clontech, Palo Alto, CA ('Human cytokine mapping amplimers'), and PCR amplification was done according to the manufacturer's recommendation. Primer pairs specific for RB (sense: nucleotides 5′-1059-1078-3′, antisense: 5′-1966-1944-3′; stretching a 907 bp fragment of RB gene), cdc2 kinase (sense: 5′-276-296-3′, antisense: 5′-877-857-3′; 601 bp), p53 (sense: 5′-433-452-3′; antisense: 5′-1016-997-3′; 583 bp) and c-myc (sense: 5′-1015-1035-3′, antisense: 5′-3509-3489-3′; 1117 bp) were custom designed and synthesized by Bio-Synthesis, Denton, TX. Also, specific primer pairs for E7 genes of HPV 6, 11 and 16 were also designed and synthesized. After agarose gel electrophoresis, the fragments were transferred and fixed to a Hybond N^+ filter (Amersham, Arlington Heights, IL) by capillary transfer, and the filters were hybridized with the appropriate [^{32}P]γ-ATP labeled oligonucleotide probes under conditions recommended by the manufacturer. After autoradiography the films were analysed by densitometry (Fisher FB910 densitometer connected with a Hewlett Packard 3394A integrator) to obtain the target gene/G3PDH expression ratios. When possible, data obtained from RT-PCR experiments were also confirmed by conventional Northern blot analysis (data not shown).

For Western immunoblotting condyloma tissues or cell proteins were lysed at 4°C in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 0.25 M NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 50 μg/ml aprotinin and 1 mM PMSF). The lysates were clarified by centrifugation (4°C, 20000 × *g*, 5 min) and protein contents were determined by standard Bio-Rad methods. Equal amounts of lysates (100 μg) were electrophoresed on 7.5% (RB) or 12% (cdc2p34, c-myc TGFβ1 and E7) SDS-PAGE gel (Protean Minigel system, Bio-Rad), and the proteins were blotted to Hybond-ECL nitrocellulose membrane (Amersham, Arlington Heights, IL) at 30 V for 20 h in blotting buffer (192 mM glycine, 25 mM Tris, pH 8.3 and 20% methanol). The membranes were blocked in TBST (10 mM Tris-HCl, pH 8.8, 150 mM NaCl, 0.05% Tween-20) containing 5% dried milk for 1 h and then incubated 1 h at room temperature with TBST with dried milk to which monoclonal RB (IF8), cdc2 p34, c-myc (Santa Cruz Biotechnology, Santa Cruz, CA), TGFβ1 (R and D Systems, Minneapolis, MN) or HPV 16 E7 (Triton Diagnostics, Almeda, CA) antibodies were added at 5 μg/ml concentration. The blots were then probed according to the ECL Western blotting protocol (Amersham) using 1:1000 diluted HRP conjugated secondary antibody (goat anti-mouse IgG,

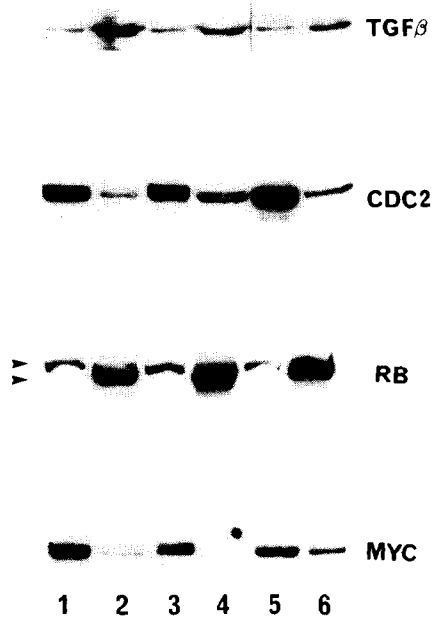

Fig. 1. Western immunoblotting patterns of three different condylomas reveals changes in protein levels of certain genes after interferon treatment. Condyloma tissues were lysed, then electrophoresed on SDS-PAGE gel, the proteins were blotted and then incubated with primary antibodies (TGFβ1, cdc2 kinase, RB and c-myc). The blots were then probed according to the ECL Western blotting protocol using HRP conjugated secondary antibody as described in Materials and Methods. [condylomas before (lanes 1,3,5) and after (lanes 2,4,6) IFN treatment; upper arrow: ppRB: approx. 116 kDa hyperphosphorylated RB protein; lower arrow: pRB: approx. 110 kDa underphosphorylated RB protein].

Santa Cruz Biotechnology or rabbit anti-chicken IgG, Jackson Immunoresearch Labs, respectively).

Results

In vivo interferon treatment raised the protein levels of TGFβ1 (397$\pm$46%) and RB (600$\pm$100%) genes in condylomas, but decreased significantly the cdc2 kinase (26$\pm$8%) and c-myc (22$\pm$9%) levels, as revealed by Western immunoblotting (Fig. 1). (Data are given as percentage mean of pre-treatment

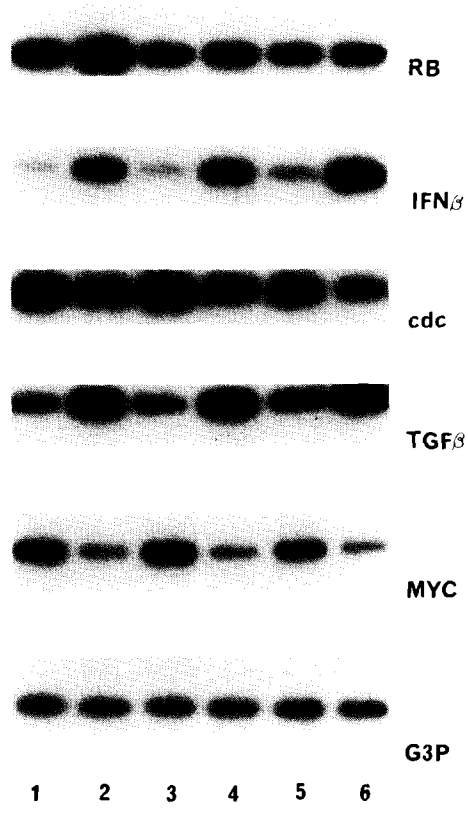

Fig. 2. Changes in mRNA levels of certain genes in different condylomas after interferon treatment support changes observed at the protein level. Total RNAs were isolated from condylomas before and after IFN treatment and subjected to reverse transcription followed by PCR amplification, using gene specific primers, according to Materials and Methods. The fragments were electrophoresed, transferred to a Hybond N^+ filter and hybridized with the appropriate [^{32}P]γ-ATP-labeled oligonucleotide probes. After autoradiography the films were analysed by densitometry. [RB: retinoblastoma gene; IFNβ: interferon β gene; cdc: cdc2 kinase gene; TGFβ: transforming growth factor β1 gene; myc: c-myc gene; G3P: glyceraldehyde-3-phosphate dehydrogenase internal control gene – condylomas before (lanes 1,3,5) and after (lanes 2,4,6) IFN treatment].

values in three different condylomas ± S.D.). In addition, Western blot experiments which measured both the hyperphosphorylated (approx. 116 kDa; ppRB, upper arrow) and underphosphorylated (approx. 110 kDa; pRB, lower arrow) forms of retinoblastoma protein, showed a change in the phosphorylation pattern of pRB shifting it toward the underphosphorylated form after interferon treatment. T24 bladder carcinoma cells were used as the positive control while WERI-Rb-1 cells served as the negative control (data not shown). To demonstrate if these changes occured at the transcriptional level, mRNA levels of those genes were also investigated in different condylomas, before and after IFN treatment. Indeed, in vivo IFN treatment raised the expression levels of TGFβ1 (207 ± 72%), IFNβ (607 ± 140%) and RB (141 ± 10%) genes in condylomas, but decreased the cdc2 kinase (60 ± 6%) and c-myc (32 ± 7%) levels (Fig. 2). These condylomas all contained 'low risk' (non-oncogenic) types of human papilloma viruses (HPV 6/11).

Since reports have demonstrated the importance of HPV type in effectiveness of IFN treatment (Schneider et al., 1987), we were also interested in the effect

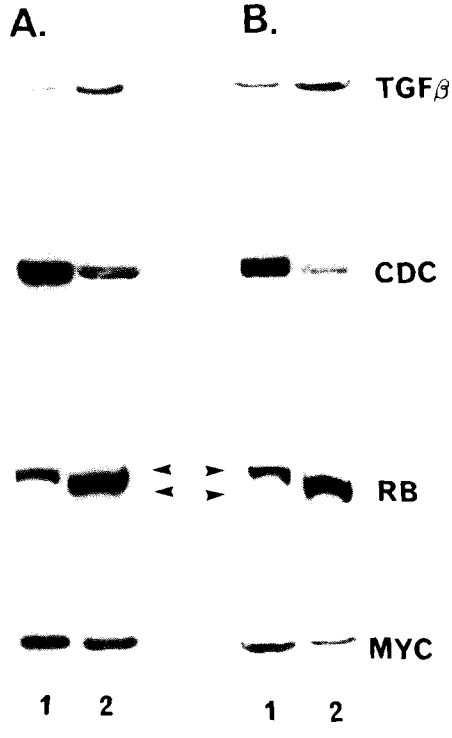

Fig. 3. Changes in protein levels of different genes in CaSki (A) and A431 (B) cells after interferon treatment. Cell lysates from CaSki (A) or A431 (B) cells were subjected to Western immunoblotting and probed with TGFβ1, cdc2 kinase, RB or c-myc antibodies (see Materials and Methods for details; data represent a typical example of three independent experiments). [lanes 1: untreated or lanes 2: IFN-treated cells, respectively].

of interferon therapy on 'high risk' (oncogenic) (HPV 16/18)-containing cells. Since biopsies from IFN treated patients carrying 'high risk' HPVs were not

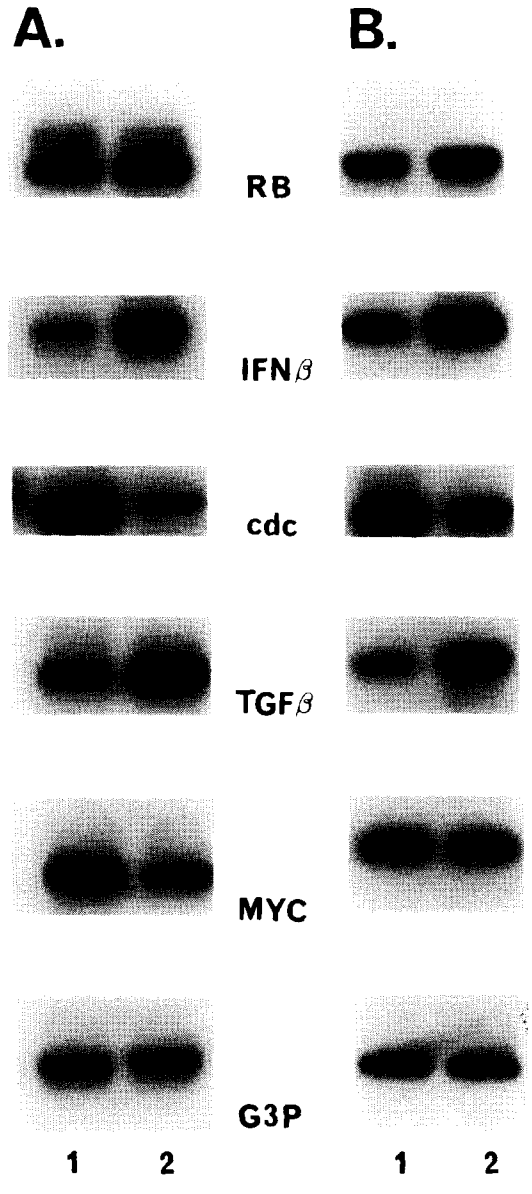

Fig. 4. mRNA expression pattern of A431 (A) and CaSki (B) cells demonstrates changes at the transcriptional level following interferon treatment. RNAs isolated from A431 (A) or CaSki (B) before (lanes 1) or after (lanes 2) IFN treatment were analyzed for expression of certain genes, as detailed in Materials and Methods (data represent a typical example of three independent experiments). [RB: retinoblastoma gene; IFNβ: interferon β gene; cdc: cdc2 kinase gene; TGFβ: transforming growth factor β1 gene; myc: c-myc gene; G3P: glyceraldehyde-3-phosphate dehydrogenase internal control gene].

available, we chose the CaSki cell line which contains a high copy number of HPV 16, and is sensitive to the growth inhibitiory effect of interferon treatment in vitro (Higashara et al., 1988). After IFN treatment a shift in the protein levels of the above mentioned genes was also observed (Fig. 3A), similar to that of the in vivo changes in 'low risk' HPV-containing keratinocytes. Characteristic increases in TGFβ1 (3-fold) and RB (2-fold) levels, but significant decreases in cdc2 kinase (4-fold) expression were detected. In vitro interferon treatment also caused a change in the phosphorylation pattern of pRB shifting it toward the underphosphorylated form. Interestingly, the extent of c-myc inhibition (20% decrease) was significantly less than in the case of 'low risk' HPV infection (60–90% decrease). These changes also occured at the transcriptional level, as demonstrated by changes in mRNA levels of the aforementioned genes as follows: RB, 1.5-fold; IFNβ, 2-fold and TGFβ1, 3-fold increases, but with a 2-fold decrease in cdc2 kinase and a 35% decrease in c-myc, respectively (Fig. 4B). The extent of changes at the mRNA level were less than at the proteins level, suggesting post-translational regulation in addition to the effect of IFN on transcription.

Using the A431 cell line (epidermoid carcinoma), in vitro IFN treatment changed the protein and mRNA levels (Fig. 3B and Fig. 4A, respectively) to a similar degree as with previous targets. Significantly increased protein levels of inhibitory genes (TGFβ1, 4-fold; RB, 2-fold) and decreased cdc2 kinase (5-fold) and c-myc (3-fold) levels were found. RNA message levels for those genes were also found to be changed significantly, a 2-fold increase in both TGFβ1 and RB, and a 3-fold increase in IFNβ but a 2.5-fold decrease in both cdc2 kinase and c-myc (Fig. 4B).

E7 mRNA and protein levels showed only moderate changes (Fig. 5B and C, 15% and 10% decrease, respectively) in oncogenic HPV-containing cells after IFN treatment in contrast with the 5-fold decrease in E7 mRNA levels found in condylomas of IFN-treated patients (Fig. 5A). Our future studies will focus on tumors carrying HPV types 16/18 to evaluate these findings in vivo.

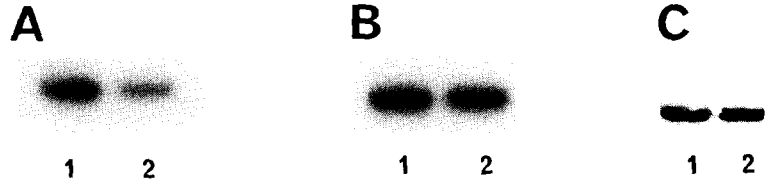

Fig. 5. mRNA and protein level of the E7 gene of human papillomavirus after interferon treatment. mRNA levels of the E7 gene were measured before (lanes 1) and after (lanes 2) interferon treatment in a representative condyloma (A) or CaSki cells (B). In addition, the level of HPV 16 E7 protein was determined by Western blotting in CaSki cells (C) before (lane 1) and after (lane 2) interferon treatment.

Discussion

Interferons are known to induce a variety of physiologic responses, including interactions with cell proliferation and differentiation (Friedman, 1989). These interactions could occur through direct effects on certain growth stimulatory genes (such as c-myc, c-fos, c-Ha-ras, etc.) or indirectly via the cytokine network (Baron et al., 1991). Keratinocyte proliferation is regulated by a cytokine/antioncogene circuit, so their involvement in IFN's antiproliferative action was anticipated.

According to our data, IFN treatment of condylomas could increase endogeneous TGFβ1 and IFNβ expression indicating the involvement of two different autocrine inhibitory pathways in IFN action. TGFβ1 as a potent inhibitor of epithelial cell proliferation (Barnard et al., 1990) suppresses c-myc mRNA and protein levels, possibly at the level of transcriptional initiation (Pietenpol et al., 1990) and could mediate the cytostatic effect of interferon treatment (Kerr et al., 1988). Indeed, a significant reduction in both mRNA and protein levels of c-myc was found in IFN-treated condyloma tissues. Different studies demonstrated a requirement for pRB in TGFβ1 suppression of c-myc transcription (Moses et al., 1990), affecting pRB synthesis and/or phosphorylation. In condylomas, in vivo IFN treatment affected both pRB synthesis and phosphorylation; increased mRNA and protein levels of RB were found together with an underphosphorylated state of pRB. This underphosphorylation could reflect the decreased levels of cdc2 kinase mRNA and protein which are believed to be involved in pRB phosphorylation (Kimchi, 1992).

The observed elevation in endogeneous IFNβ production in condylomas might represent another autocrine inhibitory loop contributing to the growth suppressive action of IFN therapy. It has been demonstrated that endogeneously produced IFNβ functions on the producer cells themselves as an autocrine growth inhibitor which is part of the mechanism controlling the decline in c-myc mRNA and the arrest of cell proliferation (Resnitzky et al., 1986).

In condylomas, suppression of the viral E7 gene has been accompanied by these observed changes (Fig. 5A). However, 'high risk' HPV (HPV-16)-containing cells were partially refractory to the interferon treatment: only a partial reduction in E7 mRNA/protein levels could be observed (Fig. 5B and C). On the other hand, elevation in TGFβ1, IFNβ and RB levels, but a decrease of cdc2 kinase expression was demonstrated, similar to changes observed in treated condylomas. In these HPV-16-containing cells the extent of c-myc suppression by IFN was less than in condylomas (Figs. 3–4.). Since the E7 oncoproteins of 'high risk' HPVs are capable of binding to the underphosphorylated RB product (Vousden, 1990) this binding could interfere with formation of the pRB/E2F complex (Pagano et al., 1992) which is believed to be responsible for regulation of several genes necessary for cell cycle progression, such as c-myc, myb, cdc2 and others (Nevins, 1992). Further, in

HPV 16/18 transformed cells TGFβ1 was unable to suppress c-myc mRNA levels, due to the association of viral oncoproteins (i.e., E7) with pRB and some additional cellular changes (Moses et al., 1992). The presence of a partial decrease in E7 mRNA/protein levels in 'high risk' HPV-containing cells after IFN, but not following TGFβ1 treatment (this latter data not shown) suggests the existence of two different, but connecting inhibitory pathways in interferon therapy. One might operate through the endogeneous TGFβ1 (as suggested by Kerr et al., 1988) and its related pathway (cdc2 kinase, pRB) to inhibit c-myc; the other via the endogeneous IFNβ circuit. The resistance of many 'high risk' HPV-containing cells to TGFβ1 treatment could be responsible for their partial refractiveness to IFN treatment. The IFNβ loop might be – at least partly – operational, but counterbalanced by the refractory TGFβ1 network. Further experiments are needed to explore these possibilities. Also, further studies are needed to assess whether higher TGFβ1 protein levels reflect the latent or the active form(s) of the secreted gene product.

Changes observed in the HPV negative epidermoid carcinoma cell line (A431) were identical to those observed in condylomas after IFN therapy suggesting a more common effect of interferons on epithelial cell proliferation which can be influenced by the presence of viral oncoproteins.

Our results provide a basis for a more complete understanding of the mechanisms of IFN action and may lead to improved treatment of human papilloma virus-related disease.

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