The effects of interferon on the expression of human papillomavirus oncogenes

Kelly Y. Kim, Lawrence Blatt† and Milton W. Taylor

Department of Biology, Indiana University, Bloomington, IN 47405, USA

Introduction

Interferons (IFNs) have been used to treat diseases caused by human papillomaviruses (HPVs), such as condyloma acuminata and cervical intraepithelial neoplasia (CIN), with mixed results [reviewed in Gross (1997) and Bornstein et al. (1993); Table 1]. The molecular basis for this discrepancy in the efficacy of IFN treatment has not been determined, but it has been observed that expression of viral oncogenes, particularly E7, is significantly higher in the nonresponders than in the responders (Arany et al., 1995); these authors tested the hypothesis that this higher expression could be due to higher viral copy numbers in the nonresponders but showed that HPV copy number had no relevance.

Some investigators have speculated that downregulation of HPV oncogene expression could be the reason for the positive clinical outcomes observed with IFN treatment (Nawa et al., 1990; De Marco & Marcante, 1993; Agarwal et al., 1994). If this is the case, an explanation for the observed discrepancy in the efficacy of IFN therapy could be that IFN is unable to suppress HPV expression universally in all patients. Variables that could contribute to this include the specific HPV type(s) responsible for the lesion, the type of IFN employed in the treatment and the presence of other aberrations unique to the infected cell, such as mutations that disrupt intracellular surveillance and regulatory mechanisms.

In the present study, we have examined the effects of the three classes of IFNs on the level of HPV gene expression in various cervical carcinoma cell lines which contain integrated copies of either HPV type 16 or HPV type 18, the two most predominant high-risk HPV types found in cervical carcinomas. In a worldwide epidemiological study consisting of 1035 cervical carcinoma patients, HPV-16 was present in 50% of the patients, HPV-18 in 14%, HPV-45 in 8%, and HPV-31 in 5% (Bosch et al., 1995). Our aim was to examine whether these IFNs, which possess distinct biological activities and potencies, would produce differential effects on viral transcription and whether HPV-16 would respond differently from HPV-18.

Methods

Cell culture and treatment. The human cervical carcinoma cell lines HeLa, C-4-II, SiHa and CaSki were purchased from the ATCC; the HPV-16-immortalized keratinocyte line, HPK1A, was generously provided by Aldo Venuti (Rome, Italy). HeLa, C-4-II, SiHa and HPK1A cell lines were maintained in Eagle’s Minimal Essential Medium (GIBCO) supplemented with 5% bovine calf serum, 100 µg/ml penicillin, 60 µg/ml streptomycin, 2.5 µg/ml fungizone and 0.29 µg/ml 1-glutamine. CaSki cells were maintained in RPMI 1640 medium (Sigma) containing the aforementioned supplements. Recombinant IFN-α2b (Intron A), human IFN-β (Frone), and rIFN-γ were generously provided by Schering (Kenilworth, NJ, USA), Serono (Italy) and Genentech (San Francisco, CA, USA), respectively. IFN was freshly diluted in the appropriate tissue culture medium immediately before use, and the cells were treated with 100 U/ml IFN for 6 or 30 h.

Isolation of total RNA from mammalian cells. Cells grown in 10 cm tissue culture plates were rinsed with PBS and lysed in 7 ml guanidinium solution (4 M guanidine thiocyanate, 20 mM sodium acetate, 0.5% Sarkosyl and 0.1 M 2-mercaptoethanol in sterile ddH₂O).
Table 1. Compilation of results from studies involving IFN treatment of CIN and cervical carcinoma

<table>
<thead>
<tr>
<th>Positive response to treatment*</th>
<th>Initial diagnosis</th>
<th>IFN type</th>
<th>Treatment route</th>
<th>Dosage</th>
<th>Duration of treatment</th>
<th>Time of final assessment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/3</td>
<td>Cervical carcinoma</td>
<td>IFN-α</td>
<td>Topical and i.m.</td>
<td>5 $\times$ 10^6 IU, daily, 3$x$10^6 IU, daily</td>
<td>15 days</td>
<td>At end of treatment</td>
<td>Garcia-Milian et al. (1996)</td>
</tr>
<tr>
<td>3/31</td>
<td>Cervical carcinoma</td>
<td>IFN-α</td>
<td>s.c.</td>
<td>10$\times$ 10^4 IU, 3 days/wk</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Wadler et al. (1995)</td>
</tr>
<tr>
<td>1/6</td>
<td>CIN II</td>
<td>IFN-α</td>
<td>i.l.</td>
<td>2 $\times$ 10^6 IU, 2 days/wk</td>
<td>5 wk</td>
<td>4 mo. after end of treatment</td>
<td>Frost et al. (1990)</td>
</tr>
<tr>
<td>0/6</td>
<td>CIN II to III</td>
<td>IFN-α</td>
<td>Topical</td>
<td>4 $\times$ 10^5 IU, 2 days/wk</td>
<td>6–8 wk</td>
<td>At end of treatment</td>
<td>Byrne et al. (1986)</td>
</tr>
<tr>
<td>0/6</td>
<td>CIN II to III</td>
<td>IFN-α</td>
<td>Topical</td>
<td>6 $\times$ 10^4 IU, 2 days/wk</td>
<td>6 wk</td>
<td>At end of treatment</td>
<td>Moller et al. (1983)</td>
</tr>
<tr>
<td>0/6</td>
<td>CIN II to III</td>
<td>IFN-α</td>
<td>Topical</td>
<td>6 $\times$ 10^4 IU, 2 days/wk</td>
<td>12 wk</td>
<td>At end of treatment</td>
<td>Moller et al. (1983)</td>
</tr>
<tr>
<td>2/5</td>
<td>CIN II to III</td>
<td>IFN-β</td>
<td>p.l.</td>
<td>2 $\times$ 10^6 IU, 2 days/wk</td>
<td>4–6 wk</td>
<td>3 mo. after end of treatment</td>
<td>Choo et al. (1986)</td>
</tr>
<tr>
<td>6/7</td>
<td>CIN II to III</td>
<td>IFN-α</td>
<td>p.l.</td>
<td>2 $\times$ 10^6 IU, 2 days/wk</td>
<td>4–6 wk</td>
<td>3 mo. after end of treatment</td>
<td>Choo et al. (1986)</td>
</tr>
<tr>
<td>6/15</td>
<td>Cervical carcinoma</td>
<td>IFN-α</td>
<td>Topical and i.m.</td>
<td>1 $\times$ 10^6 IU, daily, 2x10^6 IU, daily</td>
<td>3 wk</td>
<td>2 wk after end of treatment</td>
<td>Krusic et al. (1981)</td>
</tr>
<tr>
<td>13/26</td>
<td>Cervical carcinoma</td>
<td>IFN-α</td>
<td>s.c.</td>
<td>6 $\times$ 10^4 IU, daily</td>
<td>2–4 mo.</td>
<td>At end of treatment</td>
<td>Lippman et al. (1992)</td>
</tr>
<tr>
<td>13/21</td>
<td>CIN II to III</td>
<td>IFN-α</td>
<td>i.m.</td>
<td>3 $\times$ 10^6 IU, 3–7 days/wk</td>
<td>2 mo.</td>
<td>At end of treatment</td>
<td>Toma et al. (1996)</td>
</tr>
<tr>
<td>12/17</td>
<td>CIN II to III</td>
<td>IFN-β</td>
<td>i.l./p.l.</td>
<td>2–3 $\times$ 10^5 IU, 5 days/wk</td>
<td>2–3 wk</td>
<td>10–15 days after end of treatment</td>
<td>De Palo et al. (1985)</td>
</tr>
<tr>
<td>10/12</td>
<td>CIN II</td>
<td>IFN-β</td>
<td>i.l.</td>
<td>3 $\times$ 10^6 IU, 3–7 days/wk</td>
<td>19 days</td>
<td>60 days after end of treatment</td>
<td>Rotola et al. (1995)</td>
</tr>
<tr>
<td>21/27</td>
<td>CIN II</td>
<td>IFN-β</td>
<td>i.m.</td>
<td>2–3 $\times$ 10^6 IU, 3–7 days/wk</td>
<td>10–29 days</td>
<td>60 days after end of treatment</td>
<td>Rotola et al. (1995)</td>
</tr>
<tr>
<td>20/24</td>
<td>CIN II to III</td>
<td>IFN-γ</td>
<td>Topical</td>
<td>5–100 $\times$ 10^6 IU, daily</td>
<td>28 days</td>
<td>6 mo. after start of treatment</td>
<td>Schneider et al. (1995)</td>
</tr>
</tbody>
</table>

* Patients showing complete or partial regression/total no. of patients.
per plate. DNA was sheared by passing the lysate several times through a 21G1 needle. The lysate was then layered over 3 ml 5:7 M CsCl in a siliconized tube and centrifuged at 35000 r.p.m. in Beckman SW41 rotor for 20 h at 18 °C. The pellet was resuspended in 360 µl TES (10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 1% SDS) and ethanol precipitated. The RNA pellet was collected by centrifugation at 15000 r.p.m. for 30 min at 4 °C, resuspended in 360 µl ddH₂O, and precipitated again to eliminate residual SDS from the TES solution. Finally, the pellet was dried and resuspended in ddH₂O. All solutions and reagents, except the guanidinium solution and ethanol, had been treated with 0.2% DEPC.

**Northern blot analysis.** Samples (20 to 30 µg) of total RNA of were resolved on a 1.2% agarose formaldehyde gel and transferred overnight to Zeta-Prob membrane. The running buffer contained 0.2 M MOPS, pH 7.0, 50 mM sodium acetate and 10 mM EDTA. RNA in the gel was transferred overnight to Zeta-Prob membrane (Bio-Rad) in 10 x SSC (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0). RNA was fixed onto the membrane by UV irradiation, and then the membrane was hybridized at 65 °C overnight with 32P-labelled probe in hybridization solution containing 1 mM EDTA, 0.25 M sodium phosphate, pH 7.2, and 7% SDS. The activity of the probe was at least 106 c.p.m./ml hybridization solution. After hybridization, the membrane was washed twice at 65 °C for 30 min in 1 mM EDTA, 40 mM sodium phosphate, pH 7.2, and 5% SDS. It was then washed once or twice at 65 °C for 30 min in 1 mM EDTA, 40 mM sodium phosphate, pH 7.2, and 1% SDS. The blotted membrane was exposed to Kodak X-ray film at —70 °C for 1 to 7 days.

**Radiolabelled probes.** Radiolabelled probes for Northern blot analyses were prepared as follows. Plasmids pHPV16 and pHPV18, containing HPV-16 and HPV-18 DNA cloned in Bluescript and pBR322, respectively, were purchased from the ATCC. Subgenomic fragments corresponding to the E6 and E7 ORFs were acquired from the following restriction digests: pHPV16 was digested with EcoRI and Ncol to acquire the 1314 bp fragment between nucleotides 7453 and 863, and pHPV18 was digested with BamHI and PvuII to acquire the 732 bp fragment between nucleotides 119 and 851. Products of the digests were resolved on a 1% agarose gel, and the desired fragments were purified by forcing the gel through a 0.45 µm filter. Liquid containing the fragment was collected and extracted with phenol–chloroform (1:1) and then ethanol precipitated.

The fragments were labelled by the random primer extension method. 100 ng of DNA in 9 µl ddH₂O or TE buffer was boiled for 5 min and cooled on ice. The DNA sample was then combined with 3 µl dNTP mix (10 mM dATP, 10 mM dGTP, 10 mM dTTP), 2 µl 10 x Hexanucleotide Mix (Boehringer Mannheim), 5 µl (50 µCi) [α-32P]dCTP (Amersham) and 1 µl (2 U) Klenow enzyme (Boehringer Mannheim). The mixture was incubated at 37 °C for 1 h. The labelled fragment was purified through a NICK Column (Pharmacia Biotech).

**Results and Discussion**

**HPV oncogene expression in cervical carcinoma cell lines**

Northern blot analysis was performed to characterize the expression of HPV E6 and E7 oncogenes in several HPV-containing cell lines. HeLa and C-4II cells contain integrated HPV-18, while SiHa, CaSkI and HPK1A cells contain integrated HPV-16. All of the cell lines, with the exception of HPK1A, were derived from cervical carcinomas. HPK1A cells are foreskin keratinocytes that have been immortalized by transfection with HPV-16 (Durst et al., 1987).

In HeLa cells, two bands of transcripts were observed: a major band located slightly below the 4·9 kb mark and a minor band located slightly below the 1·9 kb mark (Fig. 1). The sizes of these bands have been reported to be 3·4 and 1·6 kb by Nawa et al. (1990) and 4·8 and 1·7 kb by Lazo (1987). Two bands of transcripts were also observed in C-4II cells. However, the major band was the lower band, and the upper band, which was very faint, was greater than 4·9 kb (Fig. 2).
Northern blots for the expression of integrated HPV-16 genome in SiHa cells produced a single dominant band greater than 4-9 kb. A possible second band was located above the 1-9 kb mark, but it was barely visible (Fig. 3). Three distinct bands of transcripts were produced in CaSki cells: a minor band located above the 4-9 kb mark, another minor band located just above the 1-9 kb mark, and a major band located just below the 1-9 kb mark (Fig. 4). Finally, in HPK1A cells two bands were observed: a minor band located above the 4-9 kb mark and a major band located above the 1-9 kb mark (Fig. 5).

No two cell lines produced Northern blots with the same pattern of band sizes and intensities. Baker et al. (1987) noted that the transcripts migrating near the 28S rRNA in SiHa cells consisted of mostly host cellular sequences and that cellular polyadenylation signals were used for these transcripts. Taking this into consideration, the fact that bands corresponding to different transcript sizes were observed in different cell lines leads us to reason that HPV integration into the host genome does not occur consistently at a unique site. Transcripts of various sizes could also be produced as a result of expression from different promoters in the HPV genome. The fact that the most intense band corresponded to the larger transcripts in some cell lines and the smaller transcripts in others suggests that regulation of HPV expression from a particular promoter and, hence, the strength of that promoter could be subject to disparate intracellular regulatory mechanisms of different cell lines.

Comparison of the effects of IFN-α, IFN-β and IFN-γ on HPV oncogene expression

IFN-α, IFN-β and IFN-γ were compared for their ability to regulate HPV expression. The levels of HPV mRNA expressing E6 and E7 oncogenes were examined 6 and 30 h after treatment of the cells with 1000 IU/ml IFN. IFN did not cause much change in the level of the smaller transcript of HPV-18 in HeLa cells (Fig. 1). With respect to the larger transcripts, all three classes of IFNs caused a decrease in the level of expression by 6 h after treatment, and this decrease appeared to persist 30 h
after treatment. Nawa et al. (1990) observed that IFN-α and IFN-γ decreased the level of this transcript even 48 h after treatment. None of the IFNs had an effect in C-4II cells (Fig. 2).

In SiHa cells, none of the IFNs produced a noticeable change in the level of HPV-16 E6 and E7 expression 6 h after treatment (Fig. 3). By 30 h, however, IFN-γ produced a marked increase in the level of these transcripts. The effects of IFNs in CaSki cells were similar to those observed in HPK1A cells. In both cell lines, only IFN-γ altered the level of the transcripts expressing E6 and E7 oncogenes. The decrease in the level of transcripts was clearly noticeable in both cell lines by 30 h after IFN treatment and was most apparent in the major band (Figs 4 and 5).

None of the three classes of IFNs exhibited universal efficacy in all five cell lines. However, there was a difference in potency between IFN-γ and the other two classes of IFNs. IFN-α and IFN-β, known as type I IFNs, had similar effects. They were able to decrease the level of HPV E6 and E7 transcripts only in HeLa cells. IFN-γ, known as type II IFN, decreased the transcript level in three of the five cell lines examined. This difference between type I and type II IFNs is not totally surprising, since the signal transduction pathways mediated by the two IFN types differ.

Clinical studies involving IFN treatment of CIN and cervical cancer have primarily focused on the effects of IFN-α and IFN-β, and studies examining the effects of IFN at the molecular level have likewise focused on IFN-α and IFN-β. Woodworth et al. (1992) used an HPV-16-immortalized cervical epithelial cell line, CX16, to test the effects of IFN-γ. They showed that while IFN-α had no effect, IFN-γ treatment for 2 days decreased E6/E7. We have shown similar results using HPV-16-immortalized keratinocytes, HPK1A.

An interesting observation made by this group regarding the efficacy of IFN-γ was that these immortalized cells had to be of early passage. Late passage cells or cells malignantly transformed by subsequent transfection with the c-Ha-ras gene were not responsive to IFN-γ. HPK1A cells, used by us in the present study, are immortalized but not tumorigenic. These cells can acquire the ability to form squamous carcinomas in nude mice after gamma-irradiation and long-term culturing in vitro (Durst et al., 1995). Our hypothesis is that IFNs can lose their ability to regulate HPV expression as the cells acquire mutations resulting in increasing loss of cellular regulatory control. As the passage number increases, the probability that the cells will acquire mutations also increases. Similarly, the number of detectable mutational events increase in parallel with the stage of tumour progression in vivo (reviewed in Hunter, 1991). In his review, Hunter (1991) suggests that multiple genetic lesions are, in fact, required for carcinogenesis. Clinical studies have shown that the success of IFN treatment decreases as the malignancy of CIN increases. In one report, 29 out of 65 CIN patients achieved remission after IFN-α treatment, but none of the 33 patients with grade IB invasive carcinomas did (Vasilyev et al., 1990). In another report, 50% of the patients with grade IA and IB carcinomas experienced some form of regression after IFN-α treatment, but no changes were observed in patients with the more advanced grade IIB carcinomas (Krusic et al., 1981). This trend could be observed even within different grades of CIN, where it has been shown that 57% of CIN II patients were cured by IFN treatment but only 20% of CIN III patients were cured (Schneider et al., 1995).

Inconsistency in clinical results is reflected in the in vitro data

Studies on the effectiveness of IFN therapy in patients with CINs and cervical carcinomas have produced highly inconsistent results (Table 1), and the conclusions regarding the efficacy of IFNs have been quite controversial. In one clinical trial, for instance, IFN-β was shown to cause complete remission in 60% of the patients diagnosed with CIN II (Rotola et al., 1995). On the other hand, Frost et al. (1990) showed that fewer than 17% of women with CIN II responded positively to IFN-α2b and declared that intralesional injection of IFN had no place in the treatment of CIN.

In order to gain an insight into the basis for this discrepancy, we compared the ability of all three classes of IFNs – IFN-α, IFN-β and IFN-γ – to regulate HPV expression in cervical carcinoma cell lines. We examined the effects of these IFNs on transcription of E6 and E7 oncogenes, which have been shown to be necessary and sufficient for cellular transformation and immortalization (Munger et al., 1989; Hudson et al., 1990). The results of our Northern blots showed that IFNs, especially IFN-α and IFN-β, were not too successful in downregulating HPV expression. None of the three classes of IFNs exhibited universal efficacy in all five cell lines. Also, the potency of IFN did not correlate with the HPV type. In other words, cells containing one HPV type did not appear to be more susceptible to the effects of IFN than cells containing another HPV type. HPV-18-containing HeLa was susceptible to the effects of all three classes of IFNs, while HPV-18-containing C-4II was not susceptible to any of the IFNs. HPV-16-containing CaSki and HPK1A were susceptible to IFN-γ, while HPV-16-containing SiHa was not. This inconsistency in the effectiveness of IFNs in vitro reflects the inconsistency in the effectiveness of IFNs as therapeutic agents for cervical cancer.

HPV transcription is dependent on a complex interaction of numerous cellular factors with each other and with responsive elements within the viral genome. In order to gain a better understanding of the mechanism of IFN effect at the molecular level, we are currently investigating the effects of IFNs on the cellular factors that are necessary for HPV transcription. It is possible that IFNs are mediating their effects by modulating the level of one or more of these factors.

This research was supported in part by a grant from Schering Oncology Biotech.
References


