Inhibition of Growth, Transformation, and Expression of Human Papillomavirus Type 16 E7 in Human Keratinocytes by Alpha Interferons

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We used a model system of normal human keratinocytes (HKc) and HKc immortalized with human papillomavirus type 16 DNA (HKc/HPV16) to investigate the effects of alpha interferons (IFN- α) on the growth of HPV16-immortalized human epithelial cells, on HPV16-mediated immortalization of normal HKc, and on HPV16 gene expression. Normal HKc and HKc/HPV16 were treated with several recombinant human IFN- α subtypes (IFN- α_B , IFN- α_D , and IFN- $\alpha_{B/D}$). These IFN- α subtypes inhibited proliferation of both normal HKc and HKc/HPV16 in a dose-dependent fashion; however, although 1,000 to 10,000 U of IFN- α per ml were required to inhibit growth of normal HKc, HKc/HPV16 were substantially growth inhibited by 100 U/ml. In addition, 100 U of IFN- $\alpha_{B/D}$ per ml inhibited transformation of normal HKc by HPV16 DNA. Northern (RNA) blot analysis showed no effect of IFN- α on the mRNA levels of the HPV16 E6 and E7 open reading frames. However, immunofluorescence studies of the HPV16 E6 and E7 proteins with anti-E6 and anti-E7 monoclonal antibodies showed significant inhibition of E7 protein expression in cells treated with IFN- α , whereas E6 protein expression was not altered. The inhibition of E7 protein expression in cells treated with IFN- α was further confirmed by Western immunoblot analysis. These results suggest that IFN- α may inhibit HPV16-mediated transformation of HKc and proliferation of HKc/HPV16 through an inhibition of HPV16 E7 protein expression.

Human papillomaviruses (HPV) are believed to play an important role in the development of anogenital cancer (47). The DNAs of HPV type 16 (HPV16), HPV18, and HPV33 and, less frequently, of other types can be found in about 90% of cervical, vulvar, and penile cancer biopsies (48) and also in metastases from cervical cancer (9, 23). HPV16 and HPV18 DNAs have been shown to efficiently immortalize human foreskin and cervical keratinocytes in tissue culture (5, 18, 31). HPV16 is the most prevalent virus among the HPVs associated with malignant genital lesions: the DNA of HPV16 has been demonstrated in 50% of all genital HPV infections (46) and in 80% of biopsies from patients with Bowen's disease and Bowenoid papulosis (17). The HPV16 open reading frames (ORF) E6 and E7 have been shown to be necessary and sufficient for immortalization of human keratinocytes (HKc) and cervical cells in culture, and their continuous expression in transformed cells is required for maintenance of the transformed phenotype (3, 14, 19, 27). The same viral genes are constantly maintained and expressed in human tumors which harbor HPV (34). Recently, HPV16 E7 alone has been shown to immortalize HKc in culture at very low frequency, while E6 alone does not (13). E7 has the ability to bind to the underphosphorylated form of the protein encoded by the retinoblastoma gene, a tumor suppressor gene which is involved in regulation of the cell cycle (6). E6 is known to interact with p53, the product of another tumor suppressor gene (42). The interaction of E6 with p53 promotes p53 degradation (33). These results suggest that the transforming role of HPV16 is mediated mainly by its E7 oncogene, with the E6 protein playing an important accessory role.

Numerous reports support the observation that interferons can be effective agents in inducing the regression or preventing the recurrence of laryngeal and genital HPV-induced lesions and have confirmed the potential value of interferon therapy for the treatment of HPV-induced diseases (1, 21, 24, 39, 41). Alpha interferons (IFN- α) have been reported to cause the regression of plantar warts (35) and laryngeal papillomas (12). Although the usefulness of interferons in the treatment of HPV-induced lesions has been documented in clinical studies, the mechanism by which these interferons are able to control HPV-induced pathology is not well understood. In vitro, mouse L-cell interferon has been reported to induce reversion of morphologic transformation and elimination of extrachromosomal viral DNA in bovine papillomavirus type 1-transformed mouse C127 cells (40). In addition, IFN- α has been reported to inhibit HPV18 gene expression in HeLa cells (28); however, little is known concerning the interaction of IFN- α and HPVs at the molecular level.

Human IFN- α consists of many subtypes, encoded by individual members of the IFN- α multigene family located on the short arm of chromosome 9 (38). The different subtypes of IFN- α differ from each other in the extent of antiviral and antiproliferative activity and in host specificity (2, 8). Several novel recombinant hybrid molecules have also been produced, some of which exhibit biological activities markedly different from those of the parental molecules (36). In this study, we used a recombinant IFN- $\alpha_{B/D}$ hybrid which contains amino acids 1 to 60 and 93 to 166 from human IFN- α_B and amino acids 61 to 92 from human IFN- α_D (26). This hybrid has a broad host range of antiviral and antiproliferative activities in plaque reduction assays of vesicular stomatitis virus and a high efficacy as an antiviral agent in at least 12 different animal species (16). In addition, IFN- $\alpha_{B/D}$

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has been shown to be highly effective in preventing viral replication and cell destruction by herpes simplex virus type 1 in human monocyte cultures and to produce therapeutic responses in various murine models of viral infection (10, 11). In this study, we examined the effects of IFN- α_B , IFN- α_D , and IFN- $\alpha_{B/D}$ on the proliferation of normal HKc and HKc immortalized with HPV16 DNA (HKc/HPV16), on HPV16 E6 and E7 expression, and on the efficiency of immortalization of normal HKc by HPV16 DNA.

MATERIALS AND METHODS

Interferon. Recombinant human IFN- α_B (CGP 34043), IFN- α_D (CGP 33982), and IFN- $\alpha_{B/D}$ (CGP 35269) were provided by Ciba Geigy, Basel, Switzerland. Lyophilized IFN- $\alpha_{B/D}$ was reconstituted in phosphate-buffered saline (PBS), aliquoted, and stored at -20°C . Stock IFN- α_B and IFN- α_D solutions were aliquoted and stored at -20°C . Each aliquot was thawed only once, and working dilutions were made in tissue culture medium just before use.

Cell culture. Normal HKc were isolated from neonatal foreskin as previously described (30, 31), except the epidermis was separated from the dermis by overnight incubation with 0.25% trypsin (GIBCO). The HKc/HPV16 lines used in this study were obtained by transfection of individual normal HKc strains with the plasmid pMHPV16d, as previously described (30, 31), or with full-length HPV16 DNA that had been linearized at the *Bam*HI site, free of vector sequences (37). Both normal HKc and HKc/HPV16 were cultured in serum-free MCDB153-LB medium (31) supplemented with hydrocortisone (0.2 μM), insulin (5 $\mu\text{g}/\text{ml}$), transferrin (10 $\mu\text{g}/\text{ml}$), triiodothyronine (10 nM), epidermal growth factor (5 ng/ml), and bovine pituitary extract (35 to 50 μg of protein per ml). This medium will be referred to as complete medium.

Clonal growth assay. Clonal growth assays were performed as previously described (29). Briefly, normal HKc (1,000 cells per dish) or HKc/HPV16 (2,000 cells per dish) were plated in 60-mm tissue culture dishes in complete medium without IFN- α . Groups of five dishes per condition were fed with 8 ml of complete medium containing different concentrations of IFN- α per dish, 24 h after plating. The cells were incubated for 9 days with no change of medium. On the 10th day, the resulting colonies were washed with PBS, fixed with methanol, and stained with Giemsa. It must be noted that in this assay, in which the cells are allowed to attach to the dishes before initiation of IFN- α treatment, plating efficiency is the same in all dishes; therefore, the area of the colonies provides a direct measure of cell proliferation. The area covered by colonies in each dish was measured with an Image analysis system consisting of a video camera connected to a computer (Image 1; Universal Imaging Co.).

Transformation assay. Equal numbers of cells (25,000 per dish) from second-passage cultures of individual normal HKc strains were plated into 35-mm dishes. Triplicate 35-mm dishes were fed with 1 ml each of medium with or without IFN- $\alpha_{B/D}$ (100 U/ml) for 24 h before transfection, and the cells were transfected overnight with Lipofectin (BRL, Life Technologies, Inc.). Transfection mixtures contained 5 μg of either pMHPV16d or control (calf thymus) DNA per plate in a total volume of 50 μl of transfection mixture per dish (25 μl of Lipofectin reagent per dish). Cultures were split 24 h after transfection into duplicate 100-mm dishes. Cells from each 100-mm dish were split once again 1:10 when confluent (about 1 week after transfection),

and they were incubated without further passaging, in the presence or in the absence of IFN- α , until calf thymus DNA-transfected cells had senesced. Cells immortalized by HPV16 DNA formed rapidly growing colonies. When negative control cells had senesced, dishes of HPV16-transfected cells were stained with Giemsa as colonies reached 4 to 8 mm in diameter (20 to 30 days after plating). The number of colonies present at this stage was taken as a measure of the efficiency of immortalization, since these colonies give rise to immortalized clones.

β -Galactosidase assay. Normal HKc were plated in 100-mm dishes and allowed to proliferate until approximately 80% confluent. Cells were treated for 24 h with and without IFN- $\alpha_{B/D}$ (100 U/ml) and then transfected (in the presence and in the absence of IFN- $\alpha_{B/D}$) with a plasmid expressing the β -galactosidase gene under the control of the cytomegalovirus immediate-early promoter (10 μg of DNA per dish), using Lipofectin reagent (100 μg per dish) (GIBCO/BRL, Life Technologies, Inc.). Following overnight transfection, IFN- α treatment was continued for an additional 48 h, and then cells were harvested and β -galactosidase activity was determined by a spectrophotometric assay (32).

Northern (RNA) blot analysis. HKc/HPV16 were plated in 150-mm dishes in complete medium without IFN- α . The next day, groups of five dishes per condition were fed with complete medium containing IFN- α_B , IFN- α_D , or IFN- $\alpha_{B/D}$ (1,000 U/ml) or with complete medium without IFN- α . RNA was extracted after 72 h of IFN- α treatment, according to the guanidine thiocyanate-cesium trifluoroacetate technique, modified as described by Yasumoto et al. (45). Electrophoresis of total RNA was performed in 1.2% agarose-2.2 M formaldehyde gels. RNA was transferred to a GeneScreen filter membrane (New England Nuclear) by electroblotting and hybridized with the 1.77-kb *Pst*I fragment of the HPV16 genome (nucleotides 7004 to 875) encompassing the full-length E6 and E7 ORFs, together with less than 150 nucleotides of the 3' end of L1 and the untranscribed upstream regulatory region. A human β -2 microglobulin cDNA probe was used as a positive control, and a human β -actin cDNA probe was used as a control for RNA loading (both probes were courtesy of Pedro Lazo). All probes were labeled by random priming (Promega). The hybridization buffer consisted of 5 $\times$ SSPE (1 $\times$ SSPE is 0.15 M NaCl-10 mM sodium phosphate [pH 7.4]-1 mM EDTA)-50% formamide-10% dextran sulfate-5 $\times$ Denhardt's solution-1% sodium dodecyl sulfate (SDS)-100 μg of yeast tRNA per ml; hybridization was conducted at 42 $^\circ\text{C}$ for 16 h. The filters were washed twice in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl-0.015 M sodium citrate)-0.5% SDS at room temperature for 30 min and once with 0.1 $\times$ SSC-0.1% SDS at 42 $^\circ\text{C}$ for 30 min. Filters were exposed to Kodak XAR 2 films with an enhancing screen at -70°C .

Immunofluorescence and ACAS analysis of E7 and E6 protein expression. HKc/HPV16 and normal HKc were plated in 35-mm dishes and cultured to 50% confluence. Groups of three dishes per condition were then treated for various times with 1,000 U of IFN- $\alpha_{B/D}$ or with various concentrations of IFN- α_B , IFN- α_D , or IFN- $\alpha_{B/D}$ for 72 h. The dishes were washed with PBS, and the cells were fixed and permeabilized with methanol at -20°C for 30 min. The cells were pretreated with 2% bovine serum albumin (BSA) for 30 min and then incubated for 30 min with 1 μg of the appropriate antibodies in 1 ml of PBS-0.2% BSA per dish. Controls for nonspecific fluorescence received PBS-BSA only. An anti-HPV16 E7 monoclonal antibody (Triton Diag-

nostics), an anti-HPV16/HPV18 E6 monoclonal antibody (Oncogene Science), and an anti-human β -actin monoclonal antibody (Amersham) were used. Plates were washed three times with PBS-0.2% BSA and then incubated for 30 min with 10 μ g of a fluorescein-labeled goat anti-mouse immunoglobulin G (heavy and light chain) (Research Plus Inc.) per plate. Cells were then washed extensively with PBS-0.2% BSA to remove nonspecifically bound secondary antibody. The fluorescence intensity per cell was quantified with an adherent cell analysis and sorting system (ACAS) consisting of a model 570 interactive laser cytometer (Meridian Instruments, Inc.) with a 5-W tunable argon laser. Scanning was performed at an excitation wavelength of 488 nm in three randomly selected areas per plate, in triplicate plates, for a total of 300 to 1,000 cells per experimental condition.

Immunoprecipitation and Western immunoblot analysis of HPV16 E7. HKc/HPV16 were plated in 100-mm dishes, cultured until about 50% confluent, and then treated for 72 h with 1,000 U of IFN- $\alpha_{B/D}$ per ml. Duplicate HKc/HPV16 dishes were cultured until confluent, then medium was replaced with MCDB153-LB lacking insulin, epidermal growth factor, and bovine pituitary extract. Incubation was continued for an additional 48 h period prior to harvesting the cells. Normal HKc were plated in 100-mm dishes and cultured until subconfluent. Cells were then washed with ice-cold PBS and lysed in 0.5 ml of lysis buffer (10 mM sodium phosphate, 0.1 M NaCl, 0.1% SDS, 0.1% Triton X-100, 0.5% sodium desoxycholate, 0.5% NaN₃, 20 μ g of aprotinin per ml, pH 7) per dish for 30 min on ice. Cell lysates were collected into centrifuge tubes, the DNA was sheared by 10 passages through a 25-gauge needle, and debris was removed by centrifugation at $15,600 \times g$ at 4°C. Protein concentrations in the lysates were determined by the BCA protein assay (Pierce). Samples were adjusted to 1 mM phenylmethylsulfonyl fluoride, and aliquots containing 2.2 mg of protein were diluted to a final volume of 1 ml with lysis buffer and incubated with 1 μ g of anti-HPV16 E7 antibody (Triton Diagnostics) per ml at 4°C overnight, with gentle shaking. Rabbit anti-mouse immunobeads (BioRad; 200 μ l per sample) were added, and incubation was carried on for 4 h at 37°C. Immunoprecipitates were washed three times in lysis buffer and denatured in 50 μ l of Laemmli loading buffer per sample for 10 min at 95°C. Samples were loaded on a denaturing polyacrylamide gel (5% stacking gel, 15% separating gel) as described by Laemmli (22). Proteins were then electrophoretically transferred to polyvinylidene difluoride membranes in carbonate buffer as described by Dunn (4). The blots were probed with the Photoblot chemiluminescence kit (GIBCO/BRL, Life Technologies, Inc.), using the anti-HPV16 E7 monoclonal antibody (Triton Diagnostics) at 1:400 dilution in Photoblot blocking buffer.

RESULTS

Growth of HKc/HPV16 is inhibited by IFN- α . To investigate the antiproliferative activity of IFN- α on HKc, normal HKc and HKc/HPV16 were grown in the absence and in the presence of different types of IFN- α in clonal growth assays. Figure 1 shows a typical clonal growth assay performed on an individual normal HKc strain and on HKc/HPV16d-2 (30). As shown in the figure, IFN- α (B/D hybrid, in this case) inhibits proliferation of both normal HKc and HKc/HPV16. However, the inhibitory effect of IFN- $\alpha_{B/D}$ is greater in HKc/HPV16 than in normal HKc, being evident at 100 U/ml, whereas 10,000 U/ml is needed to visibly inhibit growth of normal HKc. This experiment was repeated with individual

FIG. 1. Inhibition of clonal growth of normal HKc and HKc/HPV16 by IFN- $\alpha_{B/D}$. Normal HKc and HKc/HPV16 were plated in 60-mm tissue culture dishes (1,000 and 2,000 cells per dish, respectively). At 24 h after plating, cells were fed with medium containing the indicated concentrations of IFN- $\alpha_{B/D}$ hybrid. The dishes were incubated for 10 days at 37°C in a humidified CO₂ incubator, and then they were fixed and stained with Giemsa.

normal HKc strains from three different donors and HKc/HPV16d-2 (30) in the absence and in the presence of the indicated concentrations of IFN- α_B , IFN- α_D , or IFN- $\alpha_{B/D}$. The area covered by colonies in each dish was measured by computerized image analysis, and the results were expressed as a percentage of controls (Fig. 2). IFN- α_B and IFN- $\alpha_{B/D}$ inhibited growth of both normal HKc and HKc/HPV16 in a dose-dependent fashion. However, again HKc/HPV16 showed a greater sensitivity than normal HKc to the growth-inhibitory effect of these IFN- α subtypes (Fig. 2, panels B and B/D). IFN- α_D at the concentrations tested inhibited proliferation of HKc/HPV16 but not of normal HKc and was a less potent growth inhibitor than IFN- α_B or IFN- $\alpha_{B/D}$ (Fig. 2, panel D). Clonal growth assays were repeated with IFN- $\alpha_{B/D}$ (1,000 U/ml), using HKc/HPV16d-1 (31) and HKc/HPV16d-4 (30), in comparison with three additional normal HKc strains, each derived from a single individual (different from the ones shown in Fig. 2). As shown in Table 1, growth inhibition by 1,000 U of IFN- $\alpha_{B/D}$ ranged between 10 and 14% in normal HKc and between 50 and 87% in HKc/HPV16.

IFN- $\alpha_{B/D}$ inhibits immortalization of normal HKc transfected with HPV16 DNA. Normal HKc derived from a single individual were treated with or without IFN- $\alpha_{B/D}$ (100 U/ml; a dose which does not significantly affect proliferation of normal HKc) for 24 h. The cells were then transfected with HPV16 DNA (pMHPV16d) or with high-molecular-weight calf thymus DNA as the negative control. The next day, cells from each plate were passed into two 100-mm dishes and fed with or without IFN- $\alpha_{B/D}$. The cells were incubated until confluent, with medium changes every 48 h, and then were passaged once again with 1:10 splitting. Cells were then maintained in the absence or in the presence of IFN- α without further passaging until the calf thymus DNA-transfected cells senesced. Colonies of immortalized cells were fixed with methanol, stained with Giemsa, and counted. Colony number was reduced by about 70% in the dishes

FIG. 2. Inhibition of clonal growth of normal HKc and HKc/HPV16 by various types of IFN- α . Clonal growth assays were performed with normal HKc strains derived from three different individuals and with HKc/HPV16. Cells were plated in 60-mm dishes (1,000 and 2,000 cells per dish for normal HKc and HKc/HPV16, respectively) and allowed to attach for 24 h, then five dishes per condition were fed with medium containing the indicated concentrations of IFN- α_B , IFN- α_D , or IFN- $\alpha_{B/D}$. Colonies were fixed with methanol and stained with Giemsa 11 days after plating. The total area of the colonies was measured with a computerized image analysis system connected to a video camera. Results are expressed as a percentage of control. Total colony areas per dish in controls ranged from 0.5 (± 0.05) to 3.18 (± 0.18) cm².

treated with IFN- $\alpha_{B/D}$ (Fig. 3A). To ensure that the reduced number of immortalized colonies was not due to an effect of IFN- α on transfection efficiency, transfection controls with a plasmid expressing the β -galactosidase gene were performed in the presence and in the absence of IFN- $\alpha_{B/D}$ (100 U/ml) and β -galactosidase activity was assayed 48 h after transfection; this experiment showed that IFN- $\alpha_{B/D}$ at a concentration of 100 U/ml does not affect transfection efficiency of normal HKc (Fig. 3B). The immortalization experiment was repeated with two additional normal HKc strains, each derived from a single individual, with very similar results (inhibition of immortalization ranged from 70 to 85%).

HPV16 E6 and E7 mRNA expression is not inhibited by

TABLE 1. Sensitivity of normal HKc and HKc/HPV16 to growth inhibition by IFN- $\alpha_{B/D}$ (1,000 U/ml)

Cell line	Total colony area (% of control) ^a	% Inhibition
Normal HKc		
Strain K18	89 $\pm$ 8	11
Strain K22	86 $\pm$ 11	14
Strain K24	90 $\pm$ 19	10
HKc/HPV16d-1	50 $\pm$ 7	50
HKc/HPV16d-4	13 $\pm$ 2	87

^a Data are averages $\pm$ standard deviations of triplicate determinations. Clonal growth assays were performed as described in Materials and Methods, and total colony area was measured by computerized image analysis.

IFN- α . HKc/HPV16d-2 were treated with 1,000 U of the various IFN- α subtypes per ml for 72 h, and total RNA was isolated and analyzed by Northern blotting. A *Pst*I fragment of HPV16 containing the upstream untranslated region and ORF E6 and E7 was used as a specific E6/E7 probe. This probe detects the same bands (a 1.8- and a 4.2-kb band) detected in the same cell lines by polymerase chain reaction-generated probes specific for the E6 or the E7 ORF (20, 29). RNA loading was determined by probing the blot with a human β -actin cDNA probe. When corrected for RNA loading, the results showed that the expression of HPV16 E6 and E7 mRNA was not inhibited by any of the three types of IFN- α (Fig. 4). As a positive control, we hybridized the same blot with a human β -2 microglobulin cDNA. As reported by others (7, 15) β -2 microglobulin mRNA was

FIG. 3. Inhibition of HPV16-mediated transformation of HKc by IFN- $\alpha_{B/D}$. (A) Normal HKc at passage two were plated in triplicate 35-mm tissue culture dishes (25,000 cells per dish). At 24 h after plating, cells were fed with medium with or without IFN- $\alpha_{B/D}$. A day later, cells were transfected in triplicate with pMHPV16d. Negative controls were transfected with calf thymus DNA. At 24 h after transfection, cells were trypsinized and plated into two 100-mm dishes for each 35-mm dish and, when confluent, were split again 1:10 into 100-mm dishes. When negative controls had senesced, the dishes were stained with Giemsa and colonies were counted. Error bars represent standard deviations. (B) Control for effects of IFN- $\alpha_{B/D}$ on transfection efficiency. Normal HKc at passage two were plated in 100-mm dishes, treated with and without IFN- $\alpha_{B/D}$ (100 U/ml) for 24 h, and then transfected, in the presence or in the absence of IFN- α , with a plasmid expressing β -galactosidase. IFN- α treatment was continued for 48 h after transfection; cells were then harvested, and β -galactosidase activity was measured. The results are averages $\pm$ range of duplicate determinations.

FIG. 4. HPV16 E6 and E7 mRNA expression is not inhibited in the presence of IFN- α . HKc/HPV16 were plated in 150-mm dishes and allowed to grow to 50% confluence. Groups of five dishes per condition were then treated for 3 days with 1,000 U of IFN- α_B , IFN- α_D , or IFN- $\alpha_{B/D}$ per ml. Total RNA was extracted, and Northern blot analysis was conducted on 30 μ g of total RNA per lane, using probes for HPV16 E6 and E7, human β -actin, and human β -2 microglobulin.

induced by IFN- α_B and IFN- $\alpha_{B/D}$. IFN- α_D had no effect on β -2 microglobulin mRNA (Fig. 4).

IFN- α inhibits HPV16 E7 but not E6 protein expression. HKc/HPV16 were treated with IFN- $\alpha_{B/D}$ (100 and 1,000 U/ml) for 24 to 72 h. Cells were fixed with methanol, and immunofluorescence was performed in an ACAS system, using monoclonal anti-HPV16 E7 and anti-HPV16/HPV18 E6 antibodies. HPV16 E7 protein expression was inhibited by IFN- $\alpha_{B/D}$ in a dose- and time-dependent fashion (Fig. 5A). With 1,000 U of IFN- $\alpha_{B/D}$ per ml, inhibition was detectable after 24 h of treatment and reached 90% by 72 h (Fig. 5A). The same antibody produced no specific signal in normal HKc (Fig. 5B) and has been used to immunoprecipitate E7 from HPV16-immortalized human cervical cells and keratinocytes (44) and to detect E7 in Western blots (43). The fluorescence produced by the anti-E7 antibody in HKc/HPV16 was localized in the nucleus (not shown).

A control experiment using anti-human β -actin monoclonal antibodies showed that β -actin levels were not affected by a 72-h treatment with IFN- α , indicating that the inhibition of E7 protein expression was not the result of a general depression of protein synthesis by IFN- α (Fig. 5B). The results of the ACAS analysis with the anti-E7 antibody were confirmed by immunoblotting, performed with the same anti-HPV16 E7 antibody: no signal was detected in normal HKc (Fig. 6), whereas E7 levels were clearly decreased in HKc/HPV16 treated with 1000 U of IFN- $\alpha_{B/D}$ per ml (Fig. 6).

To make sure that the inhibition of E7 expression by IFN- α was not a consequence of growth arrest by IFN- α , we performed immunoblotting for E7 in HKc/HPV16 at logarithmic growth phase and after growth arrest by maintenance in growth factor-depleted medium (MCDB153-LB lacking epidermal growth factor, bovine pituitary extract, and insu-

FIG. 5. Inhibition of E7 expression in HKc/HPV16 by IFN- $\alpha_{B/D}$ hybrid. (A) HKc/HPV16 were plated in triplicate 35-mm dishes per condition per time point and treated with IFN- $\alpha_{B/D}$ at the indicated concentrations, for various times. Immunofluorescence was performed by using a monoclonal anti-HPV16 E7 antibody (Triton Diagnostics). The intensity of fluorescence per cell was quantified with an ACAS. Error bars represent standard deviations. (B) The anti-HPV16 E7 antibody was used to stain HKc/HPV16 and normal HKc, in comparison with a control for nonspecific fluorescence (F.I.C.) consisting of HKc/HPV16 treated with the fluorescein isothiocyanate-labeled secondary antibody but not with the primary anti-E7 antibody. An anti-human β -actin antibody was used to stain HKc/HPV16 treated for 72 h with or without IFN- $\alpha_{B/D}$ (1,000 U/ml). Error bars represent standard deviations.

lin) for 48 h at confluence. We found no changes in the levels of E7 protein under these conditions (data not shown).

HPV16 E6 protein levels, as measured by immunofluorescence and ACAS, were not significantly affected by a 72-h treatment with any of the IFN- α subtypes at a concentration of 1,000 U/ml (Fig. 7), indicating that the effect of IFN- α may be specific for E7. As for the anti-E7 antibody, no specific fluorescence was detected in normal HKc with the anti-E6 antibody (Fig. 7) and the fluorescent signal was localized in the nucleus (data not shown).

DISCUSSION

To measure the effects of IFN- α on cell proliferation, we used a modification of the traditional clonal growth assay used to measure proliferation of epithelial cells. In our procedure (29), the cells, plated at clonal density, are allowed to seed for 24 h before initiation of the treatment. Consequently, plating efficiency is not affected by the treat-

FIG. 6. Inhibition of E7 protein expression in HKc/HPV16 by IFN- α (Western blot analysis). Normal HKc and HKc/HPV16 plated in 100-mm dishes were treated for 72 h with IFN- α . Cells were then extracted, and equal amounts of protein per sample were immunoprecipitated with the anti-HPV16 E7 antibody (Triton Diagnostics). The immunoprecipitates were then separated by SDS-polyacrylamide gel electrophoresis, and Western blotting was performed. The blots were probed by using the Photoblot chemiluminescence kit with the HPV16 E7-specific monoclonal antibody.

ment, and the total area of the colonies becomes a direct measure of cell proliferation. Colony areas can be easily and accurately measured by computerized image analysis. The results obtained with this procedure are comparable with those obtained in growth assays performed in mass culture conditions (29). This method works very well to measure growth modulation by agents such as IFN- α , which do not produce modifications of cell shape or size. We demonstrated that HKc/HPV16 are more sensitive than normal HKc to growth inhibition by IFN- α . This increased sensitivity cannot be ascribed to differences in the proliferation rates between normal HKc and HKc/HPV16, since in these experiments we used actively proliferating normal HKc at their first or second passage, which grow at a rate similar to that of HKc/HPV16 (doubling times ranging between 24 and 36 h). In addition, although there is some variability in the growth rates of individual strains of normal HKc, their response to IFN- α in terms of percent growth inhibition was remarkably reproducible (as shown in Fig. 2, in which

FIG. 7. Treatment of HKc/HPV16 with IFN- α does not influence E6 protein levels. HKc/HPV16 in 35-mm dishes were treated with 1,000 U of IFN- α , IFN- α , or IFN- α for 72 h. Immunofluorescence and ACAS analysis were conducted in triplicate dishes per condition, using a monoclonal anti-HPV16/HPV18 E6 antibody. Error bars represent standard deviations. NHKc, normal HKc; IFC., control for nonspecific fluorescence, consisting of HKc/HPV16 treated with the fluorescein isothiocyanate-labeled secondary antibody but not with the primary anti-E6 antibody.

normal HKc data are averages $\pm$ standard deviations of at least three independent assays conducted on three individual normal HKc strains, and in Table 1, in which data are presented from three additional normal HKc strains). The three subtypes of IFN- α examined had different antiproliferative activities in both normal HKc and HKc/HPV16: IFN- α was the most potent in these assays, IFN- α was the least potent, and IFN- α had intermediate activity. These results agree with the results of previously reported studies of the relative potencies of different subtypes of IFN- α in other systems (16, 26). In all cases, though, substantially lower doses of IFN- α were required to inhibit growth of HKc/HPV16 than of normal HKc.

Recent studies describe inhibition of HPV18 E6 and E7 mRNA in HeLa cells by IFN- α and IFN- γ (28). Also, Woodworth et al. (43) reported that in HPV-immortalized human cervical cells IFN- γ transcriptionally decreases E6 and E7 expression, whereas a recombinant consensus form of IFN- α (by Amgen) does not. We found no inhibition by three distinct types of IFN- α of the expression of HPV16 mRNA in HKc/HPV16. Although the exact message(s) which codes for E6 and E7 has not been cloned and characterized in these cell lines, the pattern of expression observed with specific E6 and E7 probes indicates that these ORF are mainly present in transcripts detectable on Northern blots as two distinct bands: a 4.2-kb band, which is detected by probes for the entire early region, and a 1.8-kb band, which is also detected by all early region probes and is the major band detected by E6- or E7-specific probes. This band may contain different transcripts, all starting at P97 and differentially spliced. These are likely to include the transcript(s) coding for E7. When we used retinoic acid as a modulator of HPV16 E6 and E7 expression, we found that a decrease in the signals of these two bands (of 4.2 and 1.8 kb) corresponded to a decrease in the protein levels for E6 and E7, detected by both ACAS and Western blot analysis (20). Similarly, a decrease of these messages caused by transforming growth factor β is accompanied by a corresponding decrease in the protein levels for E6 and E7 (44), and in that case the effect of transforming growth factor β was transcriptional. Whichever the specific message(s) involved, we feel that the lack of a decrease of these bands in the presence of IFN- α can be reasonably interpreted as an indication that IFN- α does not inhibit E6 and E7 mRNA expression in these cells. The discrepancy between our results and those previously reported (28, 43) reflects profound differences in the activities of IFN- α and IFN- γ with respect to HPV gene expression and is worthy of further investigation. As a control for IFN- α activity, we used β -2 microglobulin (a protein that is part of the histocompatibility complex), which has been shown to be induced by various types of IFN (7, 15). As expected, the mRNA for β -2 microglobulin was induced by IFN- α and IFN- α . IFN- α did not produce an induction of β -2 microglobulin mRNA.

IFN- α significantly inhibited HPV16 E7 expression at the protein level, but did not appear to affect HPV16 E6 protein expression, as measured by immunofluorescence and ACAS. This result is somewhat in contrast with the data of Woodworth et al., which show no effect of a consensus IFN- α (Amgen) on E7 protein expression (43). A likely explanation for this discrepancy may again be found in the fact that we used different forms of IFN- α . In fact, the various IFN- α subtypes vary considerably in their activities. It will be of interest to directly compare these various types of IFN- α in our system. The continuous expression of the E6 and E7 ORF is necessary for the maintenance of the trans-

formed phenotype in cells transformed by HPV16 (3). We feel that the inhibition of E7 expression may determine the increased sensitivity of HKc/HPV16 to growth control by IFN- α , rather than being a consequence of it. In fact, we found that E7 protein levels were unchanged when cells were growth arrested by growth factor deprivation and confluence. At the mRNA level as well, E6 and E7 expression is not affected by growth arrest caused either by prolonged confluence or by growth factor deprivation in either HKc/HPV16 or human cervical cells (44).

We also observed that IFN- $\alpha_{B/D}$ treatment at a low dose (100 U/ml) results in a significant inhibition of transformation of normal HKc by HPV16 DNA. This result cannot be explained by an effect of IFN- α on transfection efficiency or on the expression of transfected plasmids, since the expression of β -galactosidase was the same in cells treated with this concentration of IFN- $\alpha_{B/D}$ as that in untreated cells. Whether the inhibition of transformation by IFN- α occurs merely because of a selective growth inhibition of the transformed cells or rather is mediated by a more direct effect on early events in the immortalization (perhaps by preventing viral DNA amplification or the integration of HPV DNA into the cellular genome) is the subject of current investigations. Interestingly, we have recently found that retinoic acid, which inhibits E7 (and also E6) expression at the mRNA level, almost completely abolishes HPV16-mediated immortalization of HKc at a dose (1 nM) in the physiologic range (20). It is tempting to draw a parallel among the activities of these two very different agents, which inhibit E7 expression through two different mechanisms and produce similar effects both on HKc/HPV16 proliferation (29) and on HPV16-mediated transformation (20). These observations also pose the interesting possibility that the combined use of these two agents may be advantageous in the treatment of HPV-induced lesions. The usefulness of combinations of IFN- α and 13-*cis*-retinoic acid in the treatment of cervical cancer is being explored (25). The apparent absence of inhibition of E6 protein expression (as measured by immunofluorescence and ACAS) by IFN- α would indicate that E6 and E7 are processed differently at the level of translation or perhaps differ in their turnover and its regulation. This point also constitutes an interesting subject for future studies.

The results of this study suggest that the biological mechanism by which some subtypes of IFN- α produce the regression of HPV-induced lesions may be through a specific inhibition of the expression of the HPV oncogene E7. Further studies are necessary to clarify the precise mechanism of action of IFN- α and to identify the subtypes with the highest levels of activity which may show promise for clinical applications, alone or in combination with other agents. Normal HKc and HKc/HPV16 provide an excellent model system for these studies.

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