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Response to interferon treatment decreases with epidermal dedifferentiation in condylomas

Istvan Arany[a], Miriam M. Brysk[a,b], Henry Brysk[b], Stephen K. Tyring[a,b,*]

[a]*Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, TX 77555, USA*
[b]*Department of Dermatology, The University of Texas Medical Branch, Galveston, TX 77555, USA*

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Abstract

After interferon (IFN) treatment of patients with condyloma acuminatum, groups clinically proven to be responders or nonresponders were selected, and cellular parameters that might influence the clinical response were studied in pretreatment biopsies by reverse transcription polymerase chain reaction (RT-PCR). The nonresponders were found to express higher amounts of cellular proliferative markers, such as proliferating cell nuclear antigen (PCNA), cyclin A, and *cdc*2 kinase, but lower levels of growth suppressor genes (TGF-β1, TGF-β2 and p53) before IFN treatment. The responders retained the epidermal keratinization, except for some signs of hyperproliferation (K6, K16 cytokeratins). In addition, the nonresponders showed a shift in the keratinization pattern to a mucosal or fetal type, as evidenced by high expression of the K18, K6, K16 and K13 cytokeratins but decreased K5, K14 and K10 levels before treatment. The expression of the human papillomavirus (HPV) genes is consistent with these differentiation patterns. The crucial conclusion to be drawn from this study is that those condylomas whose pretreatment phenotype most closely resembles that of normal epidermis respond to IFN treatment, whereas those more akin to nonkeratinizing epithelia fail to respond, i.e. the resistance of condylomas to IFN treatment is correlated with dedifferentiation.

Keywords: Condyloma; Human papillomavirus; Interferon treatment; Epidermal differentiation; Gene expression

1. Introduction

Infection by many types of human papillomaviruses (HPVs) has been intimately linked to the development of benign and malignant neoplasms of the genital tract (zur Hausen, 1991). HPVs interact with cellular factors to induce hyperproliferation (Arany et al., 1993) in a similar manner for nononcogenic types of HPVs, such as HPV6 and HPV11, as for their oncogenic counterparts (Ciccolini et al., 1994). In both oncogenic

* Corresponding author. Fax: + 409 7725065

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and nononcogenic HPV types, the HPV E7 gene controls cellular mechanisms in order to support viral replication and cause hyperproliferation of infected cells (Ciccolini et al., 1994). E6 and E7 are believed to lead to transformation in the oncogenic forms of HPVs (Münger and Phelps, 1993). Late genes, such as HPV L1, which encode the major viral capsid protein, are usually present in the upper epithelial layer of the epidermis, but are mostly absent in malignant lesions (Bernard and Apt, 1994).

Successful treatment of HPV-related diseases might be attained by the combined antiviral and antiproliferative effects of interferons (IFNs) on their target cells (Phelps and Alexander, 1995). Condyloma acuminatum is an HPV-induced benign lesion for which IFN treatment has often been effective, although unresponsiveness and recurrences are frequent (Cirelli and Tyring, 1994). In a clinical study of the IFN treatment of penile warts, some condylomas responded well while others did poorly (Arany et al., 1995a).

Both transcriptional and translational processes that are regulated by IFNs can be blocked by virus infection. For example, HPV proteins have been found to inactivate products of the tumor suppressor genes, RB (Barbosa et al., 1990) and p53 (Werness et al., 1990), which have been demonstrated to be involved in IFN activity (Mistchenko et al., 1993; Arany et al., 1994). Growth suppressor cytokines (TGF-β1, IFN-β) are also inhibited (Arany et al., 1993).

Following the course of IFN treatment of patients with penile warts (Arany et al., 1995a), a comparison was made of biopsies from clinical responders and nonresponders at the mRNA level, concentrating on the HPV genes. IFN response was found to be correlated with differential expression of viral genes: high levels of L1 mRNA were associated with a better IFN response, while high E7 levels predicted a poor response (Arany et al., 1995a). Studies revealed that L2 and L1 mRNAs are confined to the most differentiated cells (Stoler et al., 1989), but E6 and E7 early genes are localized to the basal cells (Iftner et al., 1992). Therefore, HPV gene expression appears to be inversely related to terminal differentiation, which is marked by expression of cytokeratins 1 and 10 as well as involucrin and filaggrin (Dürst et al., 1991). The implication of this formulation is that the epidermal keratinizing phenotype modulates the HPV gene expression.

For these reasons, the same set of biopsies are here re-examined from a complementary perspective, focusing on the correlation of IFN response with the pretreatment expression of cellular genes associated with growth suppression and with differentiation.

2. Materials and methods

2.1. Biopsy protocol

The clinical protocol pertaining to the biopsies used in this study is detailed in a companion paper (Arany et al., 1995a) and is similar to that of earlier studies. Pretreatment biopsy samples from condyloma acuminatum originating from penile skin (only genital, non-mucous membrane warts were included) were taken from patients who subsequently were determined clinically to be responders (i.e. disappearance of all clinical lesions of condyloma acuminatum) or non-responders (i.e. less than 25% reduction in the total surface area of genital warts) to a combination therapy of $\alpha + \gamma$ IFNs (Arany et al., 1994). Each group consisted of five patients. These patients were selected randomly from a larger study group after completing the clinical procedures.

Post-treatment biopsies were taken at the same sites 12 weeks after the last IFN treatment. The presence or absence of condyloma was determined by histology. Clinically normal, adjacent skin biopsies served as normal controls.

2.2. HPV detection and typing

Presence of HPVs was determined by in situ hybridization (Digene Diagnostics, Silver Springs, MD) and PCR. An L1 fragment of the HPV genome was amplified using consensus primer pairs (Manos et al., 1989). This fragment was then sequenced using type-specific primers (Rady et al., 1995). All the biopsies were determined to contain either HPV type 6 or 11 (Arany et al., 1993,

1995b). No differences were found in the occurrence or average copy numbers of these types between the responder and nonresponder groups (Arany et al., 1995b).

2.3. Isolation of RNA and quantitative analysis of mRNA levels

Total RNA was isolated from frozen biopsy samples using Tri-Reagent™ as recommended by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH). A reverse transcription polymerase chain reaction (RT-PCR) method was used to simultaneously determine different gene transcripts, as described in detail earlier (Arany et al., 1994). Primer pairs for the appropriate genes were purchased (Clontech, Palo Alto, CA) or designed (Gene Runner, Hastings Software) and synthesized. Sequences and sizes of PCR fragments are as follows: Clontech primers: G3PDH: ACCACAGTCCATGCCATCAC/TCCACCAC-CCTGTTGCTGTA—452 bp; TGF-β2: GAT-TTCCATCTACAAGACC ACGAGGGACTT-GC/CAGCATCAGTTACATCGAAGGAGAG-CCATTCG—503 bp; p53: CTGAGGTTGG-CTCTGACTGTACCACCATCC-CTCATTCAC-TCTCGGAACATCTCGAAGCG—371 bp. Designed primers: TGF-β1: TCAGAGCTCCGA-GAAGCGGT/CCGTTGTTCAGGCACTCTGG —585 bp; *cdc*2 kinase: AGTACTGCAAT-TCGGGA AATT/GGTTTCCATTTGGGAAT-GTA—601 bp; PCNA: TCCAGGGCTCCAT-CCTCAAG/CGTGCAAATTCCCAGAAGGC—418 bp; K18: CCTTCTCCACCAACTACCGG/GGAGCCCATGGATGTCGTTC—550 bp; K6: GCCGAGGAGCGTGAGCAGAT/CATTCTC-TGCTGCTGTGCGC—315 bp; K16: ATGCAG-CACCTCAGTGACCG/CAGCATCTCCAG-GTCAGTCC—357 bp; K13: CCAGCTATG-GAGGTGGTTTCG/TGCTTCAGGTGCCAG-TCACG—400 bp; K5: ATGGCTTTGGAGG-TGGTGCC/TCTCAGCTCTGAGTCCAGGC—452 bp; K14: GTGGTGGCTTTGCTGGTGGT/TCTGGCCAGGGTCAGTTCGT—390 bp; K10: CACTACTCTTCCTCCCGCAG/CCAGC-CTGGCATTGTCGATC—659 bp; INV: TGTC-CCAGCAACACACACTG/GCTGGTCTAAG-AGCTTCTTC—394 bp; FIL: GGGTCTCAT-CACAGCCACAC/CTGTCCACCAGAGGAA-GTC—449 bp. To verify the integrity of the RNA and cDNA for each experimental sample, a separate control amplification using glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) was included in the PCR run (Vowels et al., 1994). G3PDH also served as a constitutively expressed internal control, and target gene mRNA levels were normalized to the levels of G3PDH, i.e. mRNA levels are given as target gene/G3PDH ratios. Procedures for agarose gel electrophoresis, Southern transfer and hybridization have been described previously (Brysk et al., 1995b). Vimentin (dermal cell marker) mRNAs were determined in order to verify the epidermis : dermis ratios in different biopsies.

2.4. Statistical analysis

Descriptive statistical analyses and significance tests were performed by using SigmaStat for Windows 2.0 software (Jandel Scientific, San Rafael, CA).

3. Results

In conjunction with a clinical program of IFN treatment of condyloma acuminatum, we took biopsies of the lesions before treatment and also biopsies at the same sites after treatment. The patients were determined to be responders and nonresponders on the basis of the observed clinical outcome of the IFN treatment, as confirmed by histopathology. We measured mRNA levels of various markers in the biopsies in order to determine whether molecular characteristics distinguished the nonresponders from the responders.

Fig. 1 illustrates that IFN treatment upregulates growth suppressor genes and differentiation markers in the responders. The nonresponders show no substantial change (within one standard deviation (S.D.)) except that p53 is moderately downregulated. Thus, the change (or lack of change) in gene expression matches the clinical outcome for this hyperproliferative disease.

Fig. 2 compares the pretreatment mRNA levels in the responders and the nonresponders with

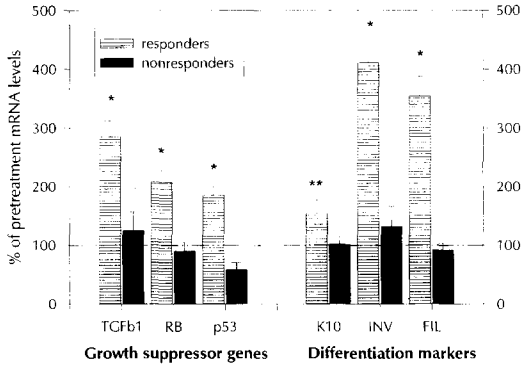

Fig. 1. Gene expression in clinically responding and nonresponding condylomas after IFN treatment. RNAs were isolated from pre- and post-treatment biopsies; mRNA levels of different genes were determined by RT-PCR. Results are expressed as percentage of untreated mRNA levels (mean ± S.D., $N = 5$). Abbreviations: TGF-β1 = transforming growth factor β1; RB = retinoblastoma antioncogene; p53 = p53 tumor suppressor gene; K10 = cytokeratin 10; INV = involucrin; FIL: filaggrin. ($*P < 0.001$; $**P < 0.05$ compared to nonresponders).

those in normal skin. Not surprisingly, all the condylomas had lower levels of the growth suppressor genes (TGF-β1, TGF-β2 and p53) than did normal epidermis. Also, growth stimulatory

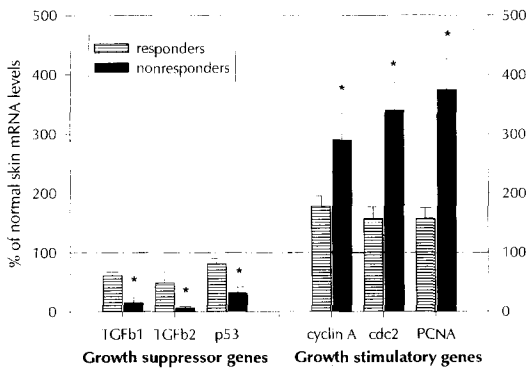

Fig. 2. Pretreatment mRNA levels of growth regulatory genes in condylomas. RNAs were isolated from pretreatment biopsies, and mRNA levels of different genes were determined by RT-PCR. Values are given as percentage of mRNA levels in normal skin (mean ± S.D., $N = 5$). TGF-β1 = transforming growth factor β1; TGF-β2 = transforming growth factor β2; p53 = p53 tumor suppressor gene; cdc2 = *cdc*2 kinase gene; PCNA = proliferating cell nuclear antigen. ($*P < 0.001$ compared to responders).

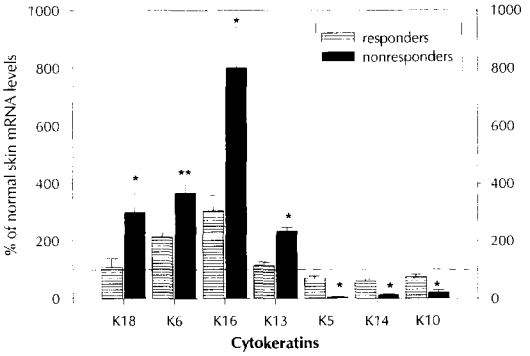

Fig. 3. Pretreatment mRNA levels of cytokeratin genes in condylomas. RNAs were isolated from pretreatment biopsies, and mRNA levels of different genes were determined by RT-PCR. Values are given as percentage of mRNA levels in normal skin (mean ± S.D., $N = 5$). Cytokeratin genes are shown in order of increasing complexity of the epithelia in which the particular keratins predominate. ($*P < 0.001$; $**P < 0.05$ compared to responders).

genes, such as cyclin A, *cdc*2 kinase and PCNA were upregulated, although nonresponders showed the most significant elevation in their mRNA levels.

Fig. 3, in the same format as Fig. 2, presents the corresponding data for keratins, arranged roughly in the order of increasing complexity of the epithelial cells in which they are normally expressed. The gene expression of the responders is fairly similar to that of normal skin, except that K6 and K16 (markers of hyperproliferation) are much enhanced whereas the epidermal keratins (K5, K14, K10) are moderately lower. In the nonresponders, the epidermal markers are drastically reduced while K13 (marker of nonkeratinizing epithelia) more than doubles; the K6, K16 pair rises further, as does K18; the pattern of change is one of hyperproliferation and dedifferentiation.

In a finer analysis of individual pretreatment samples (Fig. 4a), a strong negative correlation is found between the mRNA levels of K10, the most mature epidermal cytokeratin, and those of *cdc*2, a cell-cycle regulatory gene associated with the onset of mitosis (Dalton, 1992), i.e. the more differentiated the tissue the less the tendency to hyperproliferation. Furthermore, there is a strong

positive correlation on a case-by-case basis between the pretreatment K10 levels and the inhibition of *cdc*2 by the IFN treatment (Fig. 4b), i.e. the more keratinized the original tissue, the more successful the treatment is in turning off hyperproliferation.

Fig. 5 examines the HPV genes in the pretreatment biopsies. There is a strong negative correlation between the K10 mRNA levels and those of the E7 gene (an 'early' HPV gene which maintains

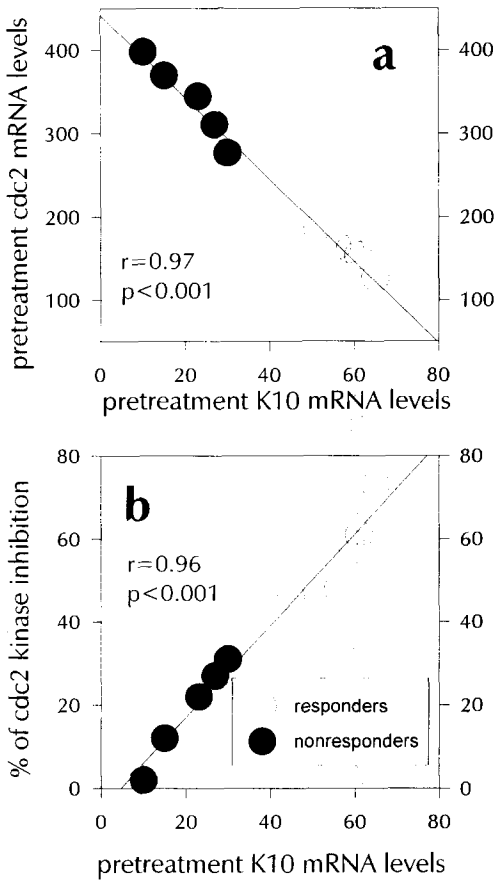

Fig. 4. Differentiation correlates with (a) proliferation and (b) IFN-mediated antiproliferative effect in individual condylomas. Status of differentiation is represented by pretreatment level of K10 mRNA in different biopsies by RT-PCR. Status of proliferation is represented by pretreatment level of *cdc*2 kinase gene. IFN-mediated antiproliferative effect is given by percent inhibition of *cdc*2 kinase transcription after IFN treatment. Correlations were analyzed by linear regression.

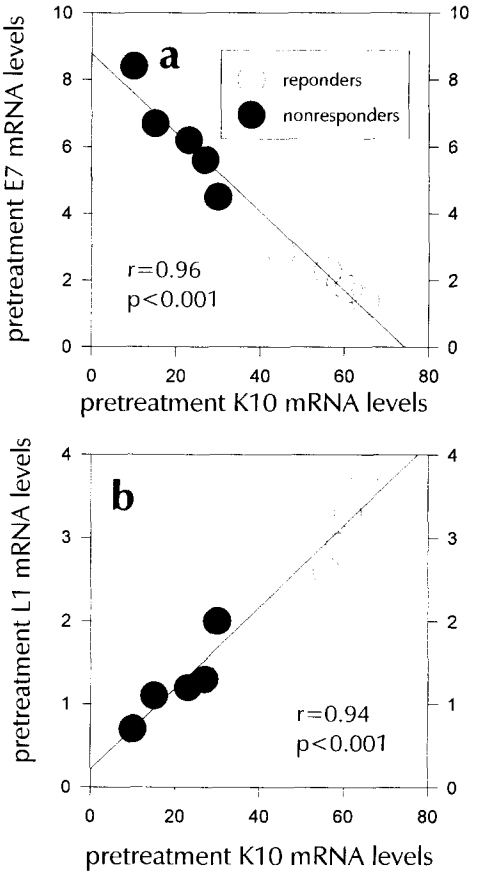

Fig. 5. Status of differentiation correlates with HPV gene transcription (a) negatively for early gene (E7) and (b) positively for late gene (L1). Status of differentiation is represented by pretreatment levels of K10 mRNA in different biopsies by RT-PCR. HPV gene mRNAs were determined in pretreatment biopsies by RT-PCR. Correlations were analyzed by linear regression.

the transformed phenotype) and a strong positive correlation with the L1 gene (the 'late' gene which encodes for major capsid proteins of the virus).

4. Discussion

IFNs are successfully used in treatment of condyloma acuminatum, although nonresponsiveness and recurrence frequently occur (Cirelli and Tyring, 1994). In a study of cultured keratinocytes from normal skin and buccal mucosa, there was a

corresponding gene upregulation by IFN-γ for the epidermal cells but not for the mucosal cells (Brysk et al., 1995a). This led us to suspect that the efficacy of IFN might depend more generally on the maturation potential of the target cell.

Figs. 2 and 4a evidenced upregulation of proliferation markers, such as *cdc*2 kinase, cyclin A and PCNAs (Demeter et al., 1994). A decreased or absent expression of the autocrine growth inhibitors TGF-βs (Yuspa, 1994), may reflect an enhanced population of proliferating cells in the nonresponder condylomas, while the responders are more differentiated and their cellular growth is more controlled. This is further evidenced in experiments shown in Fig. 4a, where we demonstrated an inverse relationship between differentiation (K10 levels) and proliferation (*cdc*2 kinase levels) in association with IFN responsiveness. Hence, the original phenotype is indicative of the receptivity to IFN treatment.

The cytokeratins are a family of proteins which form the intermediate-filament cytoskeletal network in all epithelia. Their expression varies distinctively with the cell type and, in a tissue-specific fashion, with the stage of differentiation, normal or aberrant. Cytokeratins have been studied extensively as markers of epithelial maturation and transformation (O'Guin et al., 1990). They tend to be associated in pairs (acidic and basic counterparts). In particular, the keratin pair K8, K18 is expressed in simple (non-stratified) epithelia and in certain squamous cell carcinomas. K6 and K16 are found in hyperproliferative epidermis. K4 and K13 are expressed in nonepidermal stratified epithelia such as the oral mucosa. The keratinized epidermis expresses K5, K14 in the basal cells and K1, K10 in suprabasal cells. K13, K16 and K18 are also found in fetal epidermis and in culture. Our data (Fig. 3) indicated an epidermal-type keratinization (near normal expression of K5, K14 and K10) with signs of hyperproliferation (K6 and K16) in responders. However, nonresponders showed a shift from epidermal keratinization (very low levels of K5, K14 and K10) to a mucosal/fetal type (elevated K18 and K13 mRNA levels). Some of these findings are confirmed by published immunohistochemical studies: Mullink et al. (1991) correlated the localization of HPV DNA in a number of cases of the anogenital skin with the absence of the K10 protein and the presence of K13. They commented that these results "suggest an influence of HPV infection on cellular differentiation (i.e., a shift from skin-type to mucosa-type epithelium)". However, they had no data on IFN response on those altered keratinizing condylomas. A survey of cytokeratin expression in various skin lesions (Moll et al., 1984) included the observation of a large amount of the proliferative K16 keratin in condyloma acuminatum. They devoted some attention to the fact that K1 (the pair partner of K10) is seen in some condylomas but not in others; this is compatible with the discrepancy in K10 gene expression between responders and nonresponders shown in Fig. 3. Again, no studies were done on the IFN response of those condylomas. It is noteworthy that perilesional biopsies of nonresponders do not show this shift in keratinization pattern (data not shown); these changes are therefore characteristic of the lesions only.

By in situ hybridization, the HPV activity in genital condylomata is found primarily in the upper epithelium. Stoler et al. (1989) noted that the L2 and L1 mRNAs are confined to the most differentiated cells, while they observed 'early' genes in all layers. Iftner et al. (1992) localized the E6 and E7 early genes to the basal cells; they ascribed the difference to their use of transcript-specific probes. Using cell lines, Dürst et al. (1991) concluded that "HPV gene expression appears to be inversely related to terminal differentiation, which is marked by expression of cytokeratins 1 and 10 as well as involucrin and filaggrin". The implication of this formulation is that the epidermal keratinizing phenotype modulates the HPV gene expression. Indeed, K10 levels were inversely correlated with E7 (Fig. 5a), but positively correlated with L1 levels (Fig. 5b). The more traditional perspective is that the HPV infection results in loss of the keratinizing phenotype. Both must be true, and there is feedback between the two aspects: the virus modifies the cellular RNA but is parasitically dependent on the cell for its own RNA.

The crucial conclusion from this study is that those condylomas whose phenotype most closely resembles that of normal epidermis respond to IFN treatment, whereas those more akin to nonkeratinizing epithelia fail to respond. This was evidenced in Fig. 4b, as K10 levels positively correlated with IFN-mediated antiproliferative effects. The more differentiated responders (higher K10) exerted higher IFN responses, in contrast to the nonresponders with low levels of K10. Similarly, the phenotype of cultured epidermal cells is substantially affected by exposure to IFN, while cells from buccal mucosa are essentially insensitive to it (Brysk et al., 1995a). The suggestion that the nonresponding lesional epidermal cells have evolved functionally into mucosal cells would be incorrect, however. For instance, IFN drastically downregulates K10 mRNA in normal mucosal cells and leaves p53 unchanged; in the nonresponding condylomas, K10 is unaffected and p53 downregulated (Fig. 1). Perhaps keratin expression in fetal, in contrast to adult, epidermis (Oliver, 1990) provides a closer parallel. The rule-of-thumb in cancer prognosis that dedifferentiation presages resistance to treatment also comes to mind.

Again, the connection should not be overdrawn: although the E7 protein plays a role in HPV oncogenesis, it does so only for specific types of HPV (not including the ones in this study) and through complex mechanisms (Münger and Phelps, 1993). The lesional cells of condylomas are distinct from normal cells of any type. Their responsiveness to IFN treatment deteriorates with dedifferentiation. It will be interesting to explore the extent to which an analogous rule governs IFN treatment of other cell types.

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