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Induction of apoptotic cell death by IFNβ on HPV-16 transformed human keratinocytes

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Abstract

Apoptosis, or 'programmed cell death' is a process of general biological relevance with implications in several physiological and pathological conditions of the skin. However, little is known about its induction in keratinocytes by regulator agents. In this work we demonstrate that IFNβ, but not IFNα, selectively induces programmed cell death in HPK-Ia cells, a line derived from human keratinocytes transformed with HPV-16 DNA. This IFNβ-triggered apoptosis is strictly dependent on a serum-induced partially differentiated phenotype; it occurs through the activation of a check point in the early 'S' phase, where the cells are arrested and eventually driven to apoptosis. These data indicate that apoptosis may be induced in keratinocytes by a regulator agent combined with a differentiating stimulus.

Keywords: Apoptotic cell death; Human keratinocytes; IFNβ

1. Introduction

Apoptosis, or 'programmed cell death' (Kerr et al., 1972), is an active form of cell death, distinct form necrosis, with a special impact on morphogenesis and tissue kinetics, as well as malignant growth and regression. It occurs under a variety of physiological and pathological conditions through the activation of a programmed sequence of events ultimately resulting in cell death without inflammatory reaction in the surrounding tissue. To date, a large number of apoptotic inducing stimuli have been reported, sometimes with inconsistent or conflicting effects in different cell strains or experimental settings (Razvi and Welsh, 1995). However, a few major conditions leading to apoptosis may be recognised:

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1. conflicting cellular stimuli such as differentiation commitment and mitogenic signals;
2. lack or inadequate supply of appropriate growth factors; and
3. irreparable DNA damage or genetic disregulation, allowing the cell to survive but severely impairing homeostatic control mechanisms (e.g. UV irradiation, anoxia or viral infection).

Accordingly, the following major functional roles may be attributed to this process of altruistic suicide:

1. deletion of a cell for functional reasons, as in during foetal morphogenesis or in thymocyte selection;
2. normal cell turnover and kinetic balance in proliferating tissues in adult life; and
3. suppression of a severely injured cell whose persistence may ultimately be detrimental to the organism (e.g. susceptible to originate a neoplastic growth or hosting an active viral infection) (Cotter et al., 1990; O'Brien, 1998).

Studies on the induction and role of apoptosis have mainly focused on haematopoietic and endocrine cells and cell lines. In recent years, however, the fine balance between cell proliferation and cell loss in stratified epithelia has drawn considerable attention and the study of apoptosis in epithelial cells and tissues has gained increasing interest. Consequently, apoptosis has been recognised to occur in vivo in several skin diseases (Paus et al., 1993), in HPV-induced high grade lesions in cervical mucosa (Isacson et al., 1996), and has been proposed to play a role in processes like wart regression or UV-damaged keratinocyte deletion (Henseleit et al., 1996). Nevertheless, little is known about apoptosis induction in keratinocytes, a rather resistant cell type. Indeed, deprivation of growth factors fails to induce apoptosis consistently, resulting in proliferation arrest without any evidence of cell death or toxicity. Also disappointing were the results obtained with many regulating agents and cytokines (Henseleit et al., 1996; Reinartz et al., 1996), despite most of them being well known apoptotic factors in other cell systems and potent regulators of keratinocyte response (Stadnyk, 1994). Therefore, most of the studies concerning apoptosis of keratinocytes have had to rely on induction by UV irradiation, a rather unspecific, highly toxic stress.

Interferons (IFNs) are a well known family of cytokines, with potent antiviral, immunomodulating and antiproliferative effects on several cell types (Pestka et al., 1987). They have also been implicated in apoptosis induction in a few cell lines derived from squamous cell skin carcinomas (Rodriguez-Villanueva and McDonnell, 1995) and associated with predesquamin in diploid cultured keratinocytes (Brysk et al., 1995). A novel, unusually marked cell death specifically induced by IFNβ in HPK-Ia cells has been recently reported (De Marco and Marcante, 1995). The present work aims at investigating whether this IFNβ-induced cell death could be due to apoptosis.

2. Materials and methods

2.1. Cell cultures

HPK-Ia is a cell line originating from human diploid keratinocytes in vitro transfected with human papillomavirus (HPV) type 16 genome (Dürst et al., 1987). It contains a complete transcriptionally active HPV-16 genome, integrated in a single site in an head-to-tail oligomeric fashion (Rohlfs et al., 1991). These cells do not grow at clonal density or in anchorage-independent fashion. They were propagated in Dulbecco's modified MEM supplemented with 10% FCS and sub-cultivated twice a week at a 1:2 ratio. For some experiments they were also propagated in keratinocytes serum free medium (K-SFM) as primary keratinocytes (see below). For the present work HPK-Ia between the 345th and 380th passage were used.

HaCaT is a cell line of spontaneously immortalized keratinocytes isolated from human trunk skin and known not to contain HPV (Boukamp et al., 1988). They were propagated in Dulbecco's modified MEM supplemented with 10% FCS and sub-cultivated twice a week at a 1:5 ratio.

Normal human epithelial keratinocytes (NHEK), here considered as parental cells, were isolated from neonatal or child foreskin according to an established method (Pirisi et al., 1987) and

cultivated in keratinocyte KGM medium combined with epidermal growth factor and bovine pituitary extract (Gibco Brl Life Technologies, Paisely, UK). The medium was replaced at 48-h intervals and the culture sub-cultivated at a 1:3 ratio at 90% confluence to avoid accelerated differentiation and senescence. For the present work keratinocytes between the third and eighth passage were used.

2.2. Interferons and Interferons assay

Human recombinant IFNα_{2b} (Intron A. Schering Plough S.p.A. Milan, Italy) and human recombinant IFNβ (Gibco Brl Life Technologies) were used, unless otherwise stated, at 1000 International Units (IU)/ml; human recombinant IFNγ (kindly provided by Dr V. Moriconi, Boehringer Ingelheim S.p.A., Rome, Italy) was utilised at a maximum of 100 IU/ml. Each type was reconstituted according to the suppliers' instructions, aliquoted and stored at -70°C. Each aliquot was thawed only once and working dilutions were made in tissue culture medium just before use. Antiviral activities of IFNs were determined according to the 50% virus yield reduction assay (Weigent et al., 1981).

2.3. Proliferation assay

Experiments on cell proliferation were performed as previously described (De Marco et al., 1995). Briefly, cells were plated in a 96-well plate at a density allowing an exponential growth rate for the following 5 days (10^4/well NHEK and HaCaT; 1.8×10^4/well HPK-Ia). After overnight incubation the medium was discarded and replaced with a fresh medium containing either IFNα_{2b}, IFNβ, IFNγ or a medium with no IFN. At 24-h intervals the cellular monolayers were stained with crystal violet and cell density evaluated by comparing the A_{540} of the eluted dye with a standard curve. Each condition was assayed in eight parallel replicas and the results were evaluated and plotted by the INPLOT 4 software (Graphpad Software Inc.).

2.4. DNA ladder

Cell monolayers were incubated with IFNα, β, γ or plain fresh medium. At 24, 48 and 72-h intervals the cells were washed twice with PBS, resuspended with Trypsin/EDTA and digested with 0.4% NP_{40}/0.4% Tween 20 Proteinase K and RNAse A. Cell lysates were then phenol/chloroform extracted with the 'DNA single tube' kit used according to producer's suggestions (Biotecx Laboratories Inc.). The recovered DNA was electrophoresed through a 1.2% agarose gel and visualised by ethidium bromide staining under UV transillumination.

2.5. Nucleosomes

Cells were seeded in a 96-well plate, as for the proliferation assay. After overnight incubation monolayers were washed and challenged with 100 μl of a plain complete medium or a medium containing IFNs. At subsequent 24, 48 and 72-h intervals cytoplasmic nucleosomes in adherent cells were determined, in a sandwich EIA assay, according to the 'cell death detection Elisa' kit (Boehringer Mannheim GmbH FRG). Absorbence values were compared with cell number determined in parallel cultures and results, expressed as A_{405}/A_{492} per 10^{-4} living cells, plotted by the INPLOT 4 software (Graphpad Software Inc.).

2.6. DNA synthesis assay

For synchronous DNA synthesis HPK-Ia cells were seeded at 1.8×10^4 cells/well in 96-well plates. After overnight incubation cells were washed twice with PBS, starved for 24-h in 0.1% FCS medium and then refed with 10% medium with or without 1000 IU/ml IFNβ. At intervals of 0, 8, 12, 16, 20, 24, 30, 36, 42, 48, 54, 60, 66 and 72-h after complete medium replacement, the cells were pulsed with 37 KBq of [Methyl-^{3}H] thymidine (specific activity 185 GBq (5 mCi)/mmol; Amersham, UK) for 2-h at 37°C. Cells were then washed twice with cold PBS, detached and harvested on a Skatron cell harvester. Thymidine incorporation, evaluated by liquid

scintillation, was then compared with cell number in parallel cultures and results expressed as c.p.m. per 10^5 living cells. In some experiments, thymidine incorporation was also evaluated in asynchronous cultures arranged as described for proliferation assay, and the results expressed as total c.p.m.

2.7. Cell cycle analysis

Cells were seeded in 25 cm^2 flasks, and after overnight incubation were treated with IFN-containing media or a plain medium as a control. Following incubation for various lengths of time the cells were harvested, washed twice with PBS and counted. Following centrifugation, the cells were resuspended in 100 μl of sample buffer and fixed by adding 1 ml of cold ethanol drop-wise while vortexing. Cells were left at 4°C for over 24 h. The cells were then washed twice with sample buffer and resuspended in sample buffer containing 50 μg/ml of propidium iodide (PI) and 100 U/ml of RNAse A, and incubated at room temperature for 1 h. Samples were then filtered through a 44 μm nylon mesh. Cytometric analysis and a cell count were performed with a FACScan (Becton Dikinson, San Jose, CA, USA). For each analysis, 30 000 events were recorded.

3. Results

The investigation was begun by determining the effect of IFNs on proliferation of HPV-16 transformed keratinocytes in comparison with normal diploid keratinocytes and with keratinocytes transformed by agents other than HPV. As can be seen from Fig. 1, the growth curve of HPK-Ia treated with IFNβ shows a sharp divergence from that of the control untreated cells: an initial severe growth rate impairment is followed by an active cell loss with negativation of the curve slope and net decrease of cell number, that drops, at the end point, below the number of the initial inoculum. This effect is not shared with IFNα, or with IFNγ, and appears to be restricted to HPV-bearing cells, as no similar effect is seen in control NHEK or HaCaT keratinocytes, although all three kinds of cells were fully responsive to antiviral state induction by any kind of IFN (Table 1). The observed cytopathic effect is also markedly different from the well-known, mild antiproliferative effect of IFNs. This latter effect, evident in HaCaT cells upon IFNγ treatment, consists of a partial growth rate reduction devoid of any morphological and biochemical evidence of cell toxicity (Table 1). Nonetheless, the growth rate

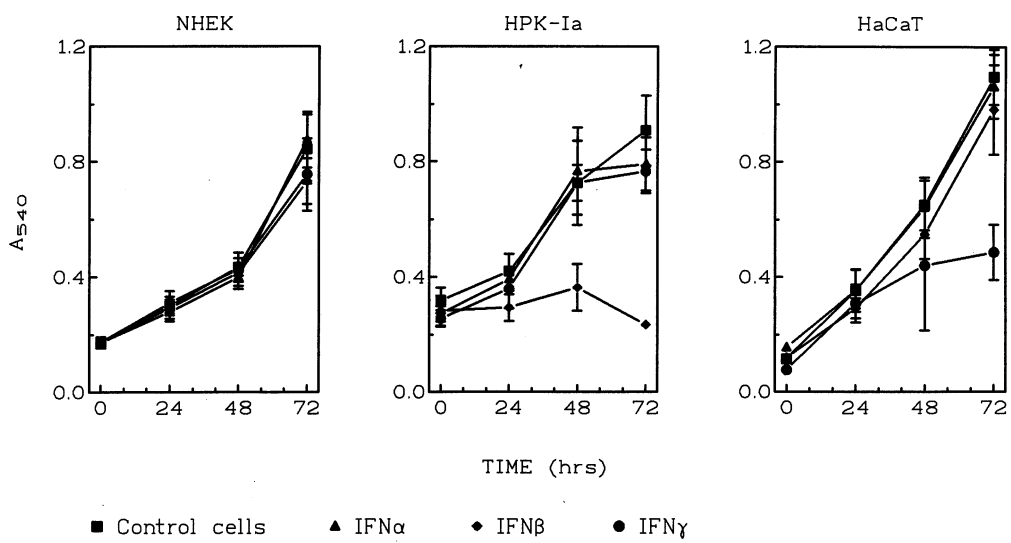

Fig. 1. Growth curves for NHEK, HPK-Ia and HaCaT keratinocytes upon IFN treatment in a representative experiment in a set of five. Bars indicate S.D.

Table 1
Effects of IFN treatment on HPK-Ia, NHEK and HaCaT keratinocytes after a 72-h treatment period[e]

	HPK-Ia/DMEM			HPK-Ia/K-SFM			NHEK			HaCAT		
	IFNα	IFNβ	IFNγ	IFNα	IFNβ	IFNγ	IFNα	IFNβ	IFNγ	IFNα	IFNβ	IFNγ
Antiviral activity[a]	+++	+++	+	+++	+++	+	+++	+++	++	+++	+++	++
Cytopathic effect[b]	−	+++	−	−	−	−	−	−	−	−	−	−
Antiproliferative activity[c]	−	NE	−	−	−	−	−	−	−	−	−	+
DNA ladder	−	+	−	−	−	−	ND	ND	ND	ND	ND	ND
Nucleosome elevation	−	+	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
Thymidine incorp.[d]	+	−	+	+	+	+	+	+	+	+	+	+
'S' phase arrest	−	+	−	−	−	−	ND	ND	ND	ND	ND	ND

[a] +++, 50% plaque yield reducing dose below 10^1 IU/ml; ++, 50% plaque yield reducing dose between 10^1 and 3×10^1 IU/ml; +, 50% plaque yield reducing dose between 3×10^1 and 10^2 IU/ml.

[b] +++, cell number below 20% of control culture and below the initial inoculum; −, cell number within 1 S.D. of control culture.

[c] +, cell number positively increased above the initial inoculum but 1 S.D. below the one of control culture; −, cell number comparable within 1 S.D. to the one of control culture.

[d] +, value comparable within 1 S.D. to the one of control culture; −, value below the basal incorporation of control cells at time 0 (i.e. in 0.1% FCS).

[e] NE, not evaluable; ND, not done.

remains positive and leads to an increase of total cell number, although to a considerably lesser extent than that of the control (Fig. 1).

In examining whether this cell death occurs via necrosis or apoptosis the authors first looked at the cell DNA fragmentation. As a matter of fact, DNA fragmentation at the linker region between nucleosomes by a specific endonuclease (Enari et al., 1998) is generally regarded as a biochemical hallmark of apoptosis (Wyllie 1980). In panel a of Fig. 2 a DNA ladder is evident in samples extracted from HPK-Ia after 48 and 72-h treatments with IFNβ, while control untreated cells do not show any DNA fragmentation. No DNA fragmentation was evident in IFNα- or γ- treated HPK-Ia cells, nor in HaCaT or NHEK cells tuated with any kind of IFN (Table 1). These results are in agreement with those obtained in proliferation assays. It was also investigated whether this fragmentation was associated with the formation of an immunologically detectable cytoplasmic DNA-histone complex (nucleosomes), a distinguishing feature of apoptotic cell death. As can be seen in Fig. 2 (panel b), IFNβ induced an increasingly marked elevation of cytoplasmic nucleosomes. The kinetics of such elevation paralleled the above mentioned viable cell decrease, and once again no effect was seen in HPK-Ia cells treated with IFNα or IFNγ, or in control untreated cells, apart from a slight elevation, detectable in any condition at the final point of cultures and probably owed to culture saturation and nutrient exhaustion in the medium.

Data already published show a partial proliferation recovery upon IFNβ withdrawal (De Marco and Marcante, 1995). This recovery was increasingly smaller with longer IFNβ exposures, indirectly suggesting that responsiveness to IFNβ-induced cell death may depend on cell cycle phase. In fact, supposing that only cells falling in a specific phase of cell cycle may be committed to apoptosis, once the IFNβ is withdrawn, the culture is composed of two subpopulations: a fraction of cells that have been exposed to IFNβ during the sensitive phase (and that will follow the apoptotic programme despite the removal of the inducer), and a fraction of cells that have been exposed when unresponsive (and therefore able to proliferate). Of course, the longer the treatment, the larger the number of cells crossing the 'window' of responsiveness along the cell cycle and undergoing apoptosis; conversely, the smaller the residual population of cells that had not crossed

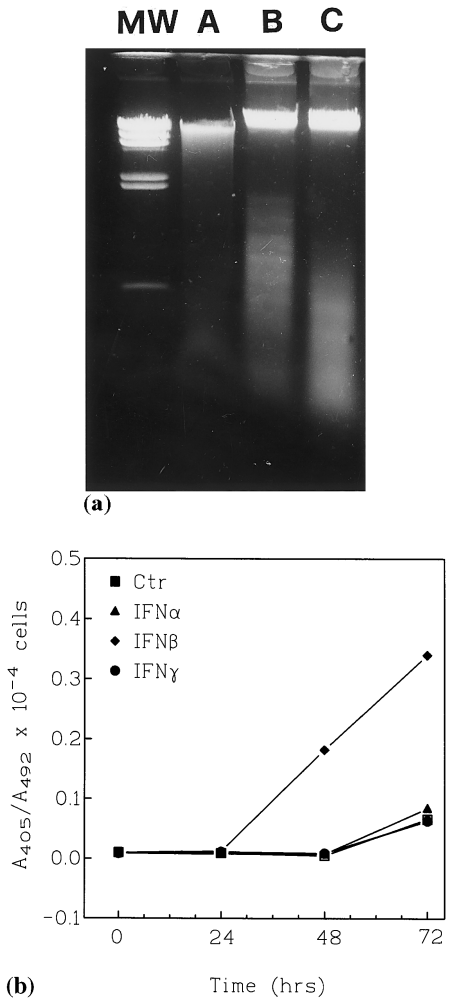

Fig. 2. Panel a: Agarose gel electrophoresis of DNA extracted from HPK-Ia control untreated cells (lane a); HPK-Ia cells treated with IFNβ for 48-h (lane b); and from HPK-Ia cells treated with IFNβ for 72-h (lane c). MW, molecular weight marker (λ DNA cleaved with Hind III) of 23130; 9416; 6557; 4361; 2322; 2027 and 567 base pair length. DNA was visualised by ethidium bromide staining and UV transillumination. Panel b: Cytosolic nucleosomes in IFN treated and control untreated HPK-Ia cells at the indicated times. A representative experiment in a set of four. Symbols indicate: square, no IFN; triangle, IFNα 10^3 IU/ml; diamond, IFNβ 10^3 IU/ml; circle, IFNγ 10^2 IU/ml.

the window of responsiveness and are still able to proliferate.

To address this point we first looked at the effects of IFN treatment on thymidine incorporation by cell cultures. Thymidine incorporation is a measure of chromosomal replication and is therefore an indication of cellular activity in the 'S' phase of cell cycle (Tamm et al., 1987). [Methyl-^{3}H] thymidine incorporation in asynchronous HPK-Ia cell culture under different IFN treatment is shown in Fig. 3 A. As can be seen, a reduction of thymidine incorporation is sharply evident as early as 24-h after IFNβ challenge. At the same time very little effect on living cell number was detectable, as evident from the growth curve (Fig. 1), with IFNβ value slightly increasing compared with time 0 and still comprised within the S.D. limits. This observation indicates that a block of DNA synthesis precedes the onset of overt anticellular effects rather than being the consequence of cell function impairment by a toxic stress. No relevant effect was seen in cells treated with IFNα, while in the same cells treated with IFNγ a mild, even reduction of absolute values was seen, with the curve maintaining the overall sigmoid profile of control cells. Thymidine incorporation was also evaluated in cell cultures synchronised in G_0/G_1 phase by serum starvation. As can be seen in Fig. 3B, at 20-h the cells treated with IFNβ incorporated only 30% of the [Methyl-^{3}H] thymidine incorporation seen in control cells. This incorporation remained consistently below that of control cells throughout the experiment. Moreover, the curve trend remained consistently negative without the typical wavy profile of cycling cells. Thus IFNβ blocks the cells at a certain point within the replicative cycle preventing thymidine incorporation. To establish the exact point at which this block occurs, the inhibitory effect of IFNβ on HPK-Ia cell cycle was further examined by flow cytometric analysis. As can be seen in cell profiles (Fig. 4) and in cell count reported in Table 2, IFNβ induced a marked depletion of G_0/G_1 phase as compared with control untreated cells. In accordance with cell death kinetics data, this reduction, already evident 24-h after the beginning of treatment, became increasingly relevant with longer treatments. An analogous reduction was also observed, although to a lesser extent, in the G_2/M compartment at 24 and 48 h. Concomitantly, a parallel increment of 'S' phase was seen. This increment is due to cells accumulating at the early part of 'S' phase, where a 'foot' became evident at 24 h. With longer incubation this 'foot' shaped into a peak, whose magnitude well accounts for the depletion of other compartments (Table 2). Taken together with data on thymidine incorporation, cell cycle analysis suggests that under IFNβ treatment cells keep cycling until they

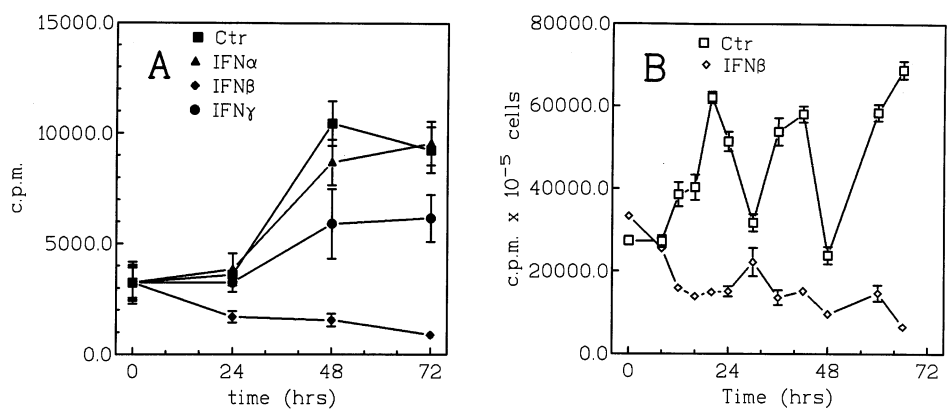

Fig. 3. [Methyl ^{3}H] thymidine incorporation by HPK-Ia upon IFN treatment. Panel A: c.p.m. mean + S.D. in asynchronous cultures. Panel B: Thymidine incorporation per 100 000 cells in cultures synchronised by serum starvation. Each panel depicts a representative experiment in a set of four.

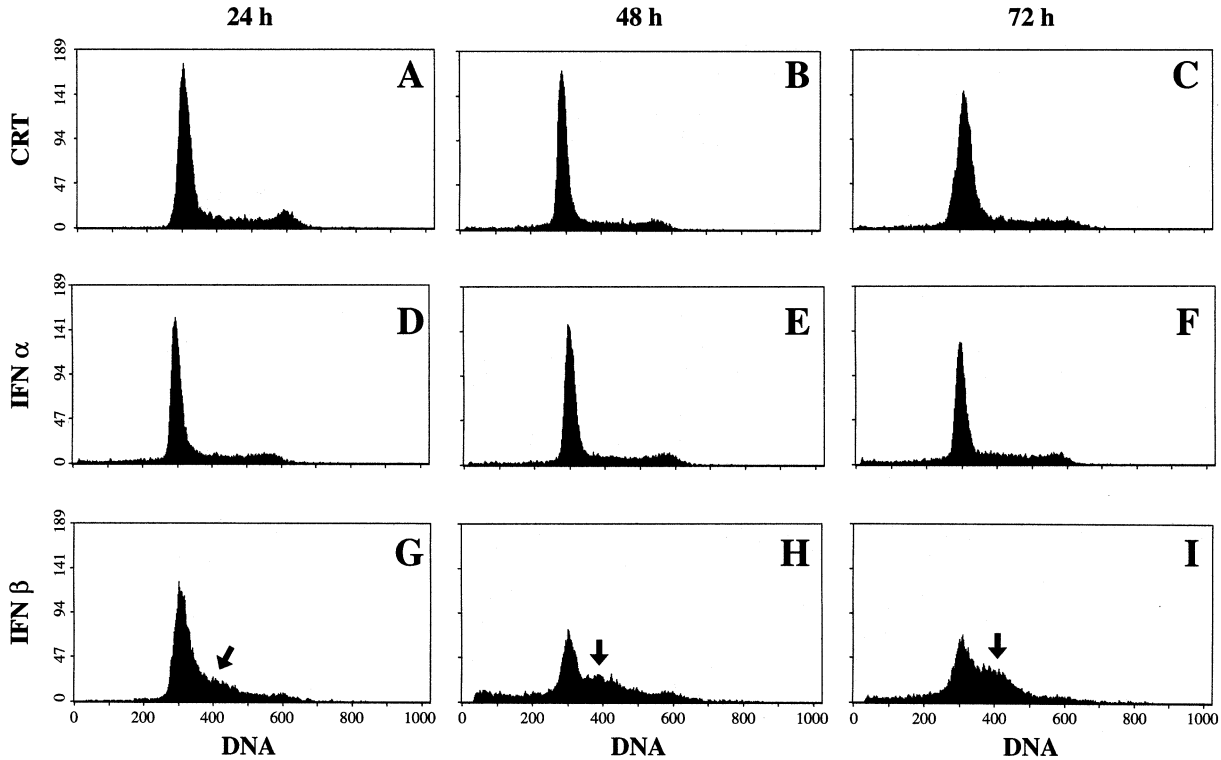

Fig. 4. Flow cytometric analysis of HPK-Ia cell cycle under IFN treatment. Panel A, B and C: Control untreated cells at 24; 48; and 72 h, respectively. Panel D, E and F: Cells treated with IFNα for 24; 48 and 72 h, respectively. A mild, uniform increase is noticed in 'S' phase. Panel G: IFNβ treated HPK-Ia at 24-h treatment. G_0/G_1 compartment is markedly reduced, the arrow indicate the slope in 'S' phase corresponding to cell cycle arrest. Panel H and I: IFNβ treated HPK-Ia at 48 and 72-h, respectively. A further reduction of the G_0/G_1 compartment is evident; cell cycle arrest in 'S' phase becomes increasingly evident as a peak.

reach a check point in the early 'S' phase where they are arrested and accumulated. The apparent contradiction between a large number of 'S' phase cells and a drop in thymidine incorporation is easily resolved on realising that these cells, included in the 'S' compartment because of their DNA content, are indeed arrested, i.e. they do not proceed in polynucleotide synthesis and do not contribute to the overall culture incorporation. Thus, reported data confirm the previously reported indirect evidence of a cycle-related effect. As expected on the basis of growth curve, no similar effect was seen in IFNα-treated cells. Here, compared with control cells only a moderate increment of 'S' phase cell percentage was observed in cell count (Table 2), in agreement with the well established, mild antiproliferative effect of IFNα, due to an elongation of 'S' phase duration (Hobeika et al., 1997).

Differentiation is a major determinant in apoptosis response (Cotter et al., 1990). In order to determine whether the IFNβ apoptotic effect is differentiation-dependent, the authors conducted parallel experiments on HPK-Ia grown in DMEM + 10% FCS and in K-SFM. In standard culture conditions, (i.e. DMEM + 10% FCS), HPK-Ia appear as partially differentiated keratinocytes with polygonal elements arranged in compact clusters with sharp irregular margins (Fig. 5, panel a). Confluence was reached by progressive expansion of clusters on interposed clear areas Fig. 5, panel b). Once cultivated in K-SFM, HPK-Ia cells regress to a basal-like phenotype with a round-ish, globose aspect (Fig. 5, panel d). Cells were arranged on a plastic surface as single elements, with no clusters; confluence was reached by increase of cell density (Fig. 5, panel e). In this

setting HPK-Ia were morphologically indistinguishable from primary basal undifferentiated keratinocytes. In these conditions, IFNβ, as well as other IFNs, did not induce any cytopathic or antiproliferative effect on cells reaching confluence (Fig. 5, panel f) in a fashion superimposable to that of control untreated cells. This 'unresponsiveness', however, appeared to be restricted to the cytopathic effect, as HPK-Ia cells remained fully responsive to each kind of IFNs as antiviral agents (Table 1), although slightly different dose response curves were observed (data not shown).

4. Discussion

IFNs are potent and pleiotropic modulators of cell growth and proliferation in a variety of cell types. The results here indicate that the recently reported cytopathic effect of IFNβ on HPV-16 transformed HPK-Ia cell line (De Marco et al. 1995) occurs with the induction of DNA ladder formation and the elevation of cytoplasmic nucleosomes. These are recognised as traits of apoptotic cell death. This apoptosis is selectively induced by IFNβ, but not by other IFNs, such as IFNα. Cells responsiveness to each kind of IFN as antiviral agent indicate that apoptosis is activated by an IFNβ-specific pathway, distinct from the one mediating the antiviral response and not shared with or cross-reactive to IFNα.

An outstanding feature of this apoptosis is represented by its massive severity involving almost the totality of cells. This is a rather uncommon finding; indeed, data reported by other authors about apoptosis on keratinocytes and keratinocyte-derived cell lines show that these cells are very resistant to apoptosis, often ranging from less than 1 to roughly 15% (Stoppler H. et al., personal communication; Jackson et al., personal communication; Brysk et al., 1995; Henseleit et al., 1996). Higher percentages are much less frequent and are achieved in HaCaT cells through highly toxic stimuli such as UV irradiation or the combination of strong anticellular drugs at full dosages (Reinartz et al., 1996). The fact that the reported effect may be elicited by an IFNβ dose within the range of those commonly used 'in vitro' indicates that the apoptotic response and its underlying mechanisms represent, at least for HPK-Ia cell line, a true pharmacological effect of IFNβ on cell growth regulation.

The activation of this apoptotic pathway, despite being distinct from the antiviral one, seems specifically linked the presence of the HPV genome. In fact, no effect was seen in parental diploid NHEK cells and, interestingly, in HaCaT cells, a line of keratinocytes transformed by agents other than HPV. These data indicate that the apoptosis response is not a consequence of the transformed state per se, but rather a specific consequence of HPV-induced transformation. Data from thymidine incorporation indicate that a sudden arrest in DNA replication occurs in IFNβ-treated cells. This arrest became evident shortly after IFNβ challenge and several hours before any cytometric evidence of cycle arrest showing an accumulation of cells at the early 'S' phase from the 24th hour onwards. Taken together these data indicate that the peak in the 'S' phase is indeed due to an arrest in DNA synthesis

Table 2
Effects of IFN treatment on HPK-Ia cells distribution along the cell cycle at indicated times[a]

	24-h			48-h			72-h		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
HPK-Ia CTR	62.2	27.4	10.4	64.8	27.5	7.8	69.7	25.8	4.5
HPK-Ia IFNα	61.6	28.1	10.3	63.5	34.0	2.5	56.2	36.1	7.7
HPK-Ia IFNβ	46.8	46.8	6.3	28.0	64.9	7.1	20.4	73.4	6.2

[a] IFNα induces a slight increment of 'S' phase; IFNβ induces a marked accumulation of cells in 'S' phase with a peak value of 73.4% at 72 h.

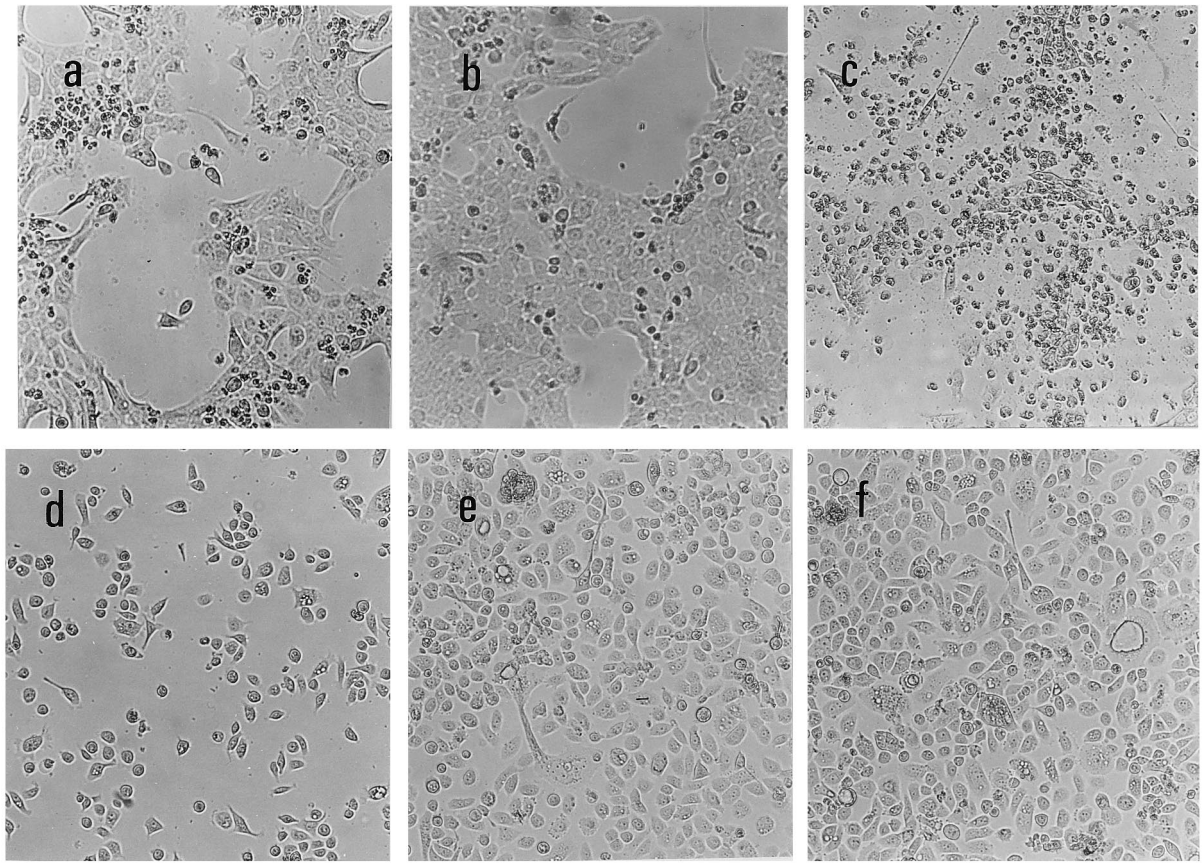

Fig. 5. Panel a: HPK-Ia at time 0 in DMEM + 10% FCS. Cells appear as polygonal elements arranged in compact cluster with sharp irregular edges. Panel b: HPK-Ia cells at time 72-h in DMEM + 10% FCS. Confluence is reached by progressive expansion of clusters on interposed clear areas. Panel c: HPK-Ia cells after a 72-h treatment with 10^3 IU/ml IFNβ: extensive cell death is evident with almost all cells as round granular elements of irregular size mostly detached from plastic. Panel d: HPK-Ia at time 0; and Panel e: at time 72 h; in keratinocyte-SFM medium. The cells display a totally different aspect from that in the presence of serum: round globose cells are evenly distributed as single elements on plastic surface and appear morphologically indistinguishable from a primary keratinocyte culture. Panel f: HPK-Ia cells in keratinocytes-SFM after a 72-h treatment with 10^3 IU/ml IFNβ. Cells show no morphological evidence of cell death appearing indistinguishable from those of control cultures.

and not to an increased number of cells cycling through the 'S' phase at a slowed rate.

It is well known that 'high risk' HPVs achieve cell transformation through the interaction of their E6 and E7 oncoproteins with the cellular p53 and RB-105 anti-oncogenic proteins. These interactions induce enzymatic degradation of the former and neutralisation of the latter (Dyson et al., 1989; Scheffner et al., 1990), abrogating the cells' ability to activate apoptosis and relieving the suppressive control on transcription initiation. This provides a most favourable cellular environment for viral replication as well as for cell transformation. IFNβ has been shown to dramatically reduce the E6/E7 transcription levels in HPK-Ia cells (De Marco and Marcante, 1995). Therefore, assuming that a consequent parallel decrease of the corresponding proteins takes place, we may hypothesise that this reduction enables the p53 and RB-105 proteins to escape from the E6/E7 functional suppression. This would in turn allow the cell to regain control on genetic integrity and, because of the alterations consequent to viral transformation, activate the apoptosis pathway.

Although many other mechanisms may also be involved, the one described above is indirectly supported by the lack of apoptosis induction in HaCaT cells, a transformed cell line known to carry a mutated form p53 protein (Lehman et al., 1993).

The IFNβ-induced apoptosis is also strictly dependent on a serum-induced partially differentiated phenotype. The presence of serum in culture medium is devoid of any modulatory effect on IFN responsiveness (as measured by its antiviral effect). This implies that IFNβ, in order to be effective, has to cooperate with other differentiation dependent cellular signals. At first glance, this is a rather surprising finding, as although a close link with differentiation is a common feature in apoptosis studies, here serum presence appears as a pro-apoptotic factor, rather than anti-apoptotic (Cotter et al., 1990). The current ideas on apoptosis, however, are very closely tailored to results coming from studies on haematopoietic cells and therefore may be not adequate to fit experimental results concerning distantly related cell types as keratinocytes. Accordingly, many biological regulators regarded as potential apoptotic inducers, on the basis of their proved effects on lympho-monocytic cells, have been already been reported to display widely divergent effects on keratinocytes (Stadnyk, 1994; Vieira et al., 1994; Henseleit et al., 1996). Serum represents a potent differentiating stimulus for 'in vitro' keratinocytes inducing primary cultures to terminal squamous differentiation and growth arrest within a few passages. A distinguishing feature of HPV-16-transformed keratinocytes is represented by their resistance to serum-induced growth arrest. HPK-Ia cells retain, at least in part, the ability to respond to differentiating stimuli modulating their phenotype. The HPV viral cycle is strictly linked to the host cell differentiation state through a fine regulation of viral transcription and expression. The early genes are highly expressed in basal and para-basal cells, while they are progressively repressed as the cells proceed toward the *stratum corneum* and terminally differentiate (Stoler et al., 1989). At the same time a corresponding late gene activation occurs with high level expression occurring in the terminally differentiated strata. The serum being a potent differentiating stimulus for 'in vitro' keratinocytes, we may postulate that its presence in the culture medium provides an inhibitory signal on E6/E7 transcription that, acting in synergism with the one brought about by IFNβ, may reduce their expression below the level needed to suppress the p53 and RB-105 effects, thus allowing the apoptotic response. In the KGM-serum free medium the cells have a basal-like undifferentiated phenotype with a concomitant fully derepressed E6/E7 expression. In these conditions no IFNβ down-regulation of E6/E7 transcription has been revealed in several RT-PCR experiments (data not shown). Accordingly, the treatment does not induce cell death, although the cell is fully responsive to IFN as antiviral agent.

In conclusion, data here reported indicate that IFNβ selectively induces programmed cell death in HPV-16 transformed keratinocytes. This IFNβ triggered apoptosis is strictly dependent on a serum-induced partially differentiated phenotype; it occurs through the activation of a check point in the early 'S' phase and may depend on the concomitant severe reduction of E6/E7 viral oncogene transcription. The mechanism of apoptosis induction by a regulatory agent combined with a differentiating stimulus is fully active also in keratinocytes although inducing stimuli may be different from those needed in other cell types.

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