Retinoic Acid and IFN Inhibition of Cell Proliferation Is Associated with Apoptosis in Squamous Carcinoma Cell Lines: Role of IRF-1 and TGase II-dependent Pathways

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Abstract
Both retinoids and IFNs are known to inhibit proliferation of many normal and transformed cells and to have an in vivo antitumor effect against a variety of cancers, including squamous cell carcinoma. Because the combination of IFNs and all-trans retinoic acid (RA) could improve their antitumor effectiveness (depending on the histological origin and state of differentiation of the cells), we compared the activity of RA and/or IFN-α2b with regard to the mechanism of growth inhibition of ME180 and SiHa cell lines, derived from squamous cervix carcinoma at different stages of differentiation. We reported previously that, in the ME180 cell line, the combined treatment significantly increased the growth inhibitory effect of the single agents. Here, we show that the SiHa cell line appears more sensitive to IFN-α2b than the ME180 cell line, and resistant to RA, which does not significantly inhibit SiHa cell growth. Induction of apoptotic cell death clearly occurs and correlates with the inhibition of cell proliferation in both cell lines. It is interesting that the induction of the transcription factor IFN regulatory factor 1 correlates with the subsequent induction of apoptosis, whereas TGase I and II expression does not. In particular, TGase I and II appear differentially expressed in the ME180 and SiHa cell lines; i.e., TGase I is expressed in ME180 and specifically inhibited by RA, whereas TGase II is expressed in SiHa. It is interesting that both IFN-α and RA are able to increase TGase II expression and activity in this cell line.

Introduction
Both IFNs and retinoids are known to regulate basic cellular functions, including growth, differentiation, and immune reactivity (1–3). They exhibit an antiproliferative effect on many normal and transformed cells and have in vivo antitumor effects against a variety of cancers (4–6). A growing body of evidence from both laboratory and clinical research now supports the concept that simultaneous exposure to both cancer chemopreventive and therapeutic agents, retinoids and IFNs, can result in enhanced antiproliferative effects compared with either agent alone in a number of malignancies or malignant cell types. Recent clinical work in advanced squamous cell carcinoma reports major activity with this regimen (7). New therapeutic paradigms involving the manipulation of normal physiological growth regulatory mechanisms, such as terminal cellular differentiation or programmed cell death, are being explored. In vitro studies have demonstrated that the combination of retinoids and IFNs resulted in additive or synergistic effects on the growth and differentiation of several SCC lines, which are thought to be the direct or indirect result of changes in gene expression (8–10). Strong evidence exists to support a major role for nuclear retinoid receptors, which transactivate the expression of target genes by binding to specific retinoic acid response elements (11), in mediating the effects of retinoids on gene expression and thereby altering the growth and differentiation of both normal and tumor cells. IFNs exert their biological activities on target cells by inducing a number of effector genes (ISGs3; Ref. 12), the transcriptional activity of which is transiently increased after the binding of type I (α and β) or type II (γ) IFNs with their specific cell-surface receptors. The transcriptional stimulation is mediated by pre-existing proteins that become activated and function as transcriptional factors that recognize an enhancer element (ISRE for type I IFN or γ-activated sequence for type II IFN) within the regulatory sequence of target genes.

Several studies have also been performed on the control of tumor cell differentiation pathways by using both IFNs and retinoids. In particular, spontaneous and drug-induced epithelial cell differentiation have been associated with the

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3 The abbreviations used are: ISG, IFN-stimulated gene; ISRE, IFN-stimulated response element; TGase, transglutaminase; RA, retinoid acid; SCC, squamous carcinoma cell; IRF-1, IFN regulatory factor 1; 2-5A, 2′-5′ oligoadenylate; RAR, RA receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
activity of a group of Ca\(^{2+}\)-dependent enzymes, i.e., TGases. These enzymes are capable of catalyzing the formation of cross-links between polypeptide chains (13). TGase I is induced during squamous differentiation and plays a role in the formation of the cross-linked envelope (14), whereas the function of TGase II is less well established. This enzyme has been implicated in the activation of several cytokines (15, 16), and the expression of TGase II has been found in association with the active, genetically controlled process of cell death called apoptosis (17–19), which occurs in the elimination of cells during morphogenesis in embryonic development, as well as in many adult tissues and in tumor growth (20). All-trans RA has been reported to induce TGase II expression and apoptosis in a variety of cell types (21, 22). The role of IFN in inducing this phenomenon has yet to be elucidated (23).

The present investigation was designed to study the mechanisms of growth inhibition exerted by IFN-α2b and RA and their combination in cultured SCCs via the analysis of their growth features, expression of differentiation markers (TGase I and II), and IFN-induced genes suggested to be involved in the negative control of cell growth (i.e., 2-5A synthetase and IRF-1). Two different cell lines were used, ME180 and SiHa, belonging to the same histotype but differing in their differentiative stage. In particular, ME180 was derived originally from an omental metastasis of a rapidly spreading cervical carcinoma, whereas SiHa was established from a primary tissue sample of a squamous carcinoma of the cervix. Our results provide evidence indicating that both agents, IFN and RA, are capable of inducing apoptosis in SCCs, IFN-α being more active than RA in inducing this phenomenon. IRF-1 expression, but not TGase II expression, correlates with the RA- and IFN-induced apoptosis phenomenon.

**Results**

**Growth Analyses**

**Antiproliferative Effects.** We reported previously (10) that RA and IFN-α2b inhibited proliferation of ME180 cells in a dose- and time-dependent manner. A markedly increased growth inhibitory effect was observed when combination treatment was carried out. In all of the combination treatments performed, even those in which low concentrations of the single agents did not significantly affect growth, a signif-

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*Fig. 1. Effects on SiHa cell growth of RA, IFN-α2b, and their combination. Cells were treated with different concentrations of RA (10\(^{-6}\) and 10\(^{-5}\) μM) and IFN-α2b (100, 500, 1000, and 2000 IU/ml) and with the combination of RA (10\(^{-6}\) μM) plus IFN-α2b at 100 IU/ml. Control and treated cells were counted daily. The results of a representative experiment are presented as mean values of viable cells counted in duplicate dishes.*
significant inhibition (up to 50%) of the proliferation rate was observed.

Fig. 1 (left) shows that IFN-α2b was a potent inhibitor of SiHa cell proliferation, whereas all-trans RA was a poor inhibitor. IFN-α2b inhibited proliferation of the SiHa cell line in a dose- and time-dependent manner. A marked growth inhibitory effect was observed when IFN-α was given at 2000 IU/ml, but a significant inhibitory effect already appeared at 100 IU/ml. In contrast, RA was unable to inhibit the growth of SiHa cells by more than 20% at a concentration of 10⁻⁶ M, which was effective in reducing cell growth in ME180 cells (10). To analyze whether combination treatment resulted in augmentation of the growth inhibitory effects, we treated the cells with a combination of suboptimal concentrations of IFN-α2b and RA (right). This combination was as effective in growth inhibition as IFN-α2b alone. Thymidine incorporation analyses confirmed the observed effects (data not shown).

**Apoptosis Analyses.** Several investigations have described the involvement of apoptosis in the growth inhibition effect induced by IFNs or RA (21-23) and have suggested a role for TGase II in apoptosis (18, 19). To examine whether, under our conditions, the observed antiproliferative effects correlated with the induction of apoptosis, we performed morphological and DNA fragmentation analyses.

**Morphological Studies.** In consideration of the results reported above, analysis was carried out to detect: (a) whether the cells detached from the substrate underwent cell death by apoptosis; and (b) whether a different behavior would be found between the two cell lines considered. In fact, these cell lines belong to the same histotype but show different cytological features. These are related to the morphological characteristics of the tumors from which they are derived, which could be responsible for the different susceptibility to RA and IFN in terms of viability. Separate analyses were first carried out in spontaneously detached and adhering cells by means of morphological methods. A different “baseline” can in fact be observed in the supernatants of the two cell lines (i.e., SiHa and ME180 untreated cells, respectively; Figs. 2a and 3a). Numerous typical mitotic figures were detectable in the supernatants of both untreated cell lines. However, few apoptotic cells were also present in ME180 untreated cells. Moreover, SiHa and ME180 cells showed a different susceptibility to the treatment with RA and IFN. ME180 cells underwent massive apoptotic cell death after treatment with either RA or IFN. Both agents were capable of inducing a significant level of programmed cell death (Fig. 3, b and c). The phenomenon was much more extensive when the two
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DNA Fragmentation Studies. As shown in Fig. 5 (left), the analysis shows that RA and IFN-α used as single agents were able to induce the formation of the typical pattern of internucleosomal endonucleolytic DNA degradation, a biochemical hallmark of apoptosis, in ME180 cells after 48 h of treatment. The pattern of fragmented DNA also appears increased by the combined treatment. DNA ladders were also evident after IFN-α treatment of SiHa cells, whereas they were not observed in cells treated with RA alone in agreement with the pattern of growth inhibition observed in Fig. 1. The observed discrete bands show analogous molecular weights (100–1000 Kb) in both cell lines.

Fig. 3. Fluorescence microscopy. Evaluation of apoptosis in ME180 cells collected from the supernatants of cultures exposed to RA (10⁻⁶ M; b), IFN-α2b (2000 IU/ml) (c), and the combination of RA and IFN-α2b (d) for 48 h. Apoptotic, fragmented nuclei are detectable from supernatants. In particular, chromatin clumps (arrows), as well as condensed nuclei with aggregated chromatin (asterisk), are observable. Note that the number of detached cells undergoing apoptosis is remarkable in ME180 cells if compared with SiHa (d).

Fig. 4. Evaluation of apoptosis in SiHa and ME180 cultured cells. The high susceptibility to apoptosis of the latter is well evident. The average values of experiments performed in quadruplicate are shown. Variations between each experiment were <10%. *, P < 0.05; **, P < 0.01. NS, not significant.
Molecular Mechanisms

Differential Expression and Regulation of TGase I and II. The cell lines ME180 and SiHa were examined for the expression of the enzymes TGase I and II. TGase assays were performed on the particulate and soluble fractions (see "Materials and Methods") to determine the activity of type I and II TGase, respectively. We observed that the two cell lines differ in the basal activity of these enzymes; only ME180 cells show high levels of TGase I activity, whereas both cell lines express similar low basal levels of TGase II (Fig. 6a). This background level corresponds to a total absence of detectable TGase II mRNA and protein in both cell lines (see control values in Fig. 6, b and c, for SiHa; data not shown for ME180). Treatment of ME180 cells with RA strongly reduced the activity of TGase I (Fig. 6a, left). IFN was unable to modulate this enzymatic activity in ME180 cells (data not shown). In SiHa cells, the addition of RA caused an increase of the enzymatic activity of TGase II (Fig. 6a, right). Surprisingly, we found that IFN-α treatment was also able to increase TGase II activity, and the combined IFN-α/RA treatment caused an additional increase, which appears more than additive with respect to the single agent treatment (Fig. 6a). A correspondent increase in the levels of TGase II protein was revealed by immunoblot analysis performed on SiHa cell extracts using a TGase II-specific antibody (Fig. 6b). As shown in the figure, an increase in TGase II occurred after 12–72 h of treatment and consistently appeared higher in the combined treatment.

Northern blot analysis performed on total RNA, fractionated and hybridized to a 32P-labeled mouse TGase II cDNA probe, parallels the described up-regulation of TGase II mRNA levels. The autoradiogram in Fig. 6c shows that IFN-α and RA act in a different time-dependent manner to increase the mRNA level that was already markedly increased after 3 h of treatment. The induction of TGase clearly occurs until 48–72 h of treatment with a significantly higher level when IFN and RA are combined.

Expression of IFN-inducible Genes. To evaluate the molecular basis of the effect of IFN and its combination with RA, we analyzed, in SiHa, the expression of IFN-induced genes that were observed to be modulated by RA in ME180 cells (10). We first analyzed the expression of 2-5A synthetase, a specific IFN-inducible gene that has been proposed to have a role in the growth inhibitory action of IFN (12). 2-5A polymersizes ATP into a series of 2'-5'-linked oligomers of the series ppp(A2'p)ₙA, which specifically activates a 2-5A-dependent RNase to cleave ribosomal and mRNAs. Northern blot analysis (Fig. 7) performed using human cDNA 9–21 (24) as a probe shows that the 2-5A synthetase gene was induced by IFN-α2b in SiHa cells and appeared highly expressed after 6 h of treatment, until 48 h. The gene was clearly not induced by RA treatment, and the observed IFN-induction remained unchanged when the cells were treated with the combination of the two agents. RA did not have any inductive or potentiating effect on the IFN inductive action of the 6-16, ISG-54, and HLA-A2 genes (data not shown).
IRF-1 Expression. It is well known that IFN-stimulated genes are characterized by the presence, in their promoter, of a highly conserved region of 12–15 bp, the ISRE, which functions as a binding site for different transcription factors, i.e., ISGF3, the transcription factor specifically formed after type I IFN treatment of cells (12, 25), and IRF-1. The specific nuclear factor IRF-1 is able to bind independently to a core domain of the ISRE present within the promoter regions of IFN-α and IFN-β genes, as well as to the ISRE present in type I IFN-inducible genes (25), thus regulating both type I IFN and IFN-induced gene expression. In addition to regulating the IFN system, IRF-1 manifests tumor suppressive activities (26) and is required for the induction of apoptosis in a different specific system (27). In ME180 cells, we previously found expression and a significant IFN-α inducibility of IRF-1. It is interesting that RA appeared to transiently induce IRF-1 gene expression, and the induction appears higher in the combined treatment (Fig. 8; Ref. 10). We found that IRF-1 was constitutively expressed at low levels also in SiHa and, when these cells were treated with IFN-α, a significant induction of expression was observed (Fig. 8). In contrast to ME180, when SiHa cells were treated with RA, no induction of IRF-1 expression was observed. Also in the combined treatment, no increase of IFN-α induction was observed.

Discussion

The mechanisms that mediate the clinically beneficial effects of IFN and RA in squamous cell carcinoma are not well understood. As the biological and genetic lesions of cancer
come into sharper focus (28, 29), the list of potential targets for tumor control expands to include the extracellular matrix (30), cell motility mechanisms, cell adhesion molecules, cytoskeletal network (31), and signal transduction pathways (32), as well as transcriptional (28) and translational (33) events. In addition, it is becoming more widely accepted that the process of multistep carcinogenesis involves not only disruption of normal cell growth but also frequently involves disruption of normal rates of cell death. It is interesting that there is much recent evidence indicating that oncogenes and tumor suppressor genes are able to regulate the susceptibility of tumor cells to undergo apoptotic cell death (34). One implication of these findings is the selection of an effective therapeutic intervention based on cell death inducibility by the commonly used cancer therapies (35), in light of the altered regulation of the dynamic homeostasis between growth, differentiation, and programmed cell death shown by the various malignancies.

Therefore, the effects of IFN-α and RA both in single or combined treatments were examined in two human cell lines from squamous carcinoma of the cervix, SiHa and ME180, established from a primitive cervical carcinoma and from an omental metastasis of a rapidly spreading cervical carcinoma, respectively. Our study shows that IFN-α was a potent inhibitor of SiHa cell proliferation, whereas all-trans RA was a poor inhibitor. The combination treatment was as effective in growth inhibition as IFN-α alone, showing a complete resistance of this cell line to the inhibition of proliferation mediated by RA. Previously, both agents demonstrated the ability to inhibit the growth of ME180 cells (10), and the antiproliferative effect was further increased by combined treatment (10). In this cell line, the IFN-α-induced 2-5A synthetase was increased by RA, although RA per se has no effect. We also found expression and a significant IFN-α inducibility of IRF-1, a transcription factor that binds to the DNA sequence elements found in the promoters of type I IFN and IFN-inducible genes (ISRE; Ref. 25). It is interesting that RA appeared to transiently induce IRF-1 gene expression in ME180 (10). The induction appears higher in the combined treatment, paralleling the observed inhibition of cell growth. In addition to regulation of the IFN system, it has recently been reported that IRF-1 manifests tumor-suppressive activity (26, 36). Recently, the existence of an IRF-1-dependent pathway of DNA damage-induced apoptosis in T-lymphocytes, distinct from the p53-mediated apoptotic pathway, was reported (27). Therefore, we also analyzed, in SiHa treated with IFN-α and/or RA, IFN-induced 2-5A synthetase and IRF-1 that have been suggested to play a role in the negative effect exerted by IFN on cell proliferation (26). As for 2-5A synthetase gene expression, this gene is induced by IFN-α2b and appeared highly expressed after 6 h of treatment until 48 h. The gene was clearly not induced by RA treatment, and the observed IFN-induction remained unchanged when the cells were treated with the combination of the two agents. With respect to IRF-1 gene expression, we observed only IFN-induced and not RA-induced IRF-1 expression. Thus, the resistance of the SiHa cell line to RA-induced inhibition of proliferation can be extended to all of the IFN-induced genes analyzed. Taken together, these results strengthen the correlation between induction of IRF-1 and cell growth inhibition.

TGase expression and activity were measured as common markers for squamous differentiation and programmed cell death. It is interesting that ME180 cells expressed a high level of TGase I, a squamous cell-specific gene. Treatment of these cultures with RA strongly suppressed this activity as revealed by a specific enzymatic assay. IFN-α was not able to modulate this enzymatic activity (data not shown). DNA fragmentation analysis supported by morphological analyses indicates induction of apoptosis in this cell line by both RA and IFN-α, as shown by the induction of the DNA ladder and by the appearance of cells with typical apoptotic features. The induction of apoptosis parallels the observed effect of inhibition of the proliferation due to RA and IFN in these cells. Apoptosis was increased by the combined treatment, as evaluated for both adhering and spontaneously detached cells with regard to the total cell population and by DNA
fragmentation analysis. The correlation between TGase II and induction of apoptosis described in several cell types (18–22) appears not to be confirmed in this case. In fact, we observed that no expression of TGase II was detectable in this cell line. The effect appears not to be mediated by modulation of RAR nuclear retinoid receptor expression, because in ME180 cells, which express RAR-α and RAR-γ but not RAR-β (10), RARs did not appear modulated by RA and IFN (10).

The same analysis was performed in SiHa cells. In these cells, IFN-α was able to induce, after 48 h of treatment (The analysis was performed until 96 h), the typical apoptotic pattern of internucleosomal endonucleolytic DNA degradation. DNA ladders were not observed in SiHa cells treated with RA alone. These results parallel the evidence of cell damage and death of apoptotic type obtained by morphological analyses and correlate with the inhibition of cell growth that was observed only after IFN-α treatment and not after RA treatment. The SiHa cell line showed no expression of TGase I in agreement with the less differentiated phenotype of the tumor from which the cells were established. Surprisingly, we found that both IFN-α and RA treatments were able to increase TGase II as shown by the increased mRNA level and protein level analyzed by both immunoblot analysis and enzymatic assay. The combined treatment with IFN-α and RA caused a higher induction in TGase II, which appears more than additive with respect to the single-agent treatment. SiHa untreated cells expressed RAR-α, RAR-β, and RAR-γ, and treatment with RA and/or IFN-α did not increase their expression level (data not shown).

It has been shown that squamous differentiation and programmed cell death share a common marker: formation of the cross-linked envelope, composed of covalently cross-linked proteins (37, 38). In these two representative SCCs, no direct correlation appears observable between differentiation and cell death as is the case for TGase activities. However, the inhibition of cell proliferation appears to be associated with the induction of programmed cell death. This could represent a specific pathway by which the specific treatments considered here could lead to cytotoxicity. Hindering of cell growth by both single agent or combination therapy protocols thus could be due to: (a) an inhibition of cell proliferation (cytostatic effect); and (b) an induction of programmed cell death (cytotoxic effect). The combination of these two different effects, cytostatic and proapoptotic, could lead to the powerful impairment of cell growth observed in our study. In addition, our study reports a different behavior of the two cell lines considered here, with respect to RA exposure. This could be correlated with the different morphological and ultrastructural characteristics of the cells. In fact, cell-to-cell and cell-substrate adhesion pathways appear to be significantly different in terms of capability of forming a monolayer with ultrastructural features typical of more invasive (ME180) or less invasive (SiHa) tumor phenotypes.4 The latter cell line showed, in fact, flat cells strongly adhering to the substrate and forming strict intercellular relationships, whereas the former did not. Accordingly, when RA and/or IFN-α exposure was performed, a different behavior of the two cell lines was found in that ME180 cells were more susceptible to apoptosis induction. This was paralleled by the possibility that cell anchorage could represent, per se, a mechanism inhibiting cell death (39). However, the loss of cell contact as a trigger for apoptotic cell death also has been suggested (40, 41). Hence, we hypothesize a close relationship between cell adhesion pathways and the anti-proliferative effects of the agents considered here.

In view of the results reported in our system (Fig. 8; Ref. 10), we can also speculate about the involvement of the IRF-1-dependent pathway in the observed RA and/or IFN-induced apoptosis in SCCs. In this respect, it would be interesting to investigate the IRF-1-dependent activation of endogenous genes (i.e., Ice, Waf1/p21, cyclin D1, and others; Ref. 27) that may mediate the induction of apoptosis. Newly acquired knowledge concerning the regulation of apoptotic cell death is being applied clinically. More must be learned about IFN- and RA-specific effects on differentiation in SCCs to provide a rational strategy for the differentiation-apoptosis paradigm.

Materials and Methods

Cell Culture and Treatments. The human epidermoid carcinoma cell line ME180, isolated from an omental metastasis of a rapidly spreading cervical carcinoma, was maintained in McCoy's 5a medium supplemented with 10% fetal bovine serum, previously inactivated at 56°C for 20 min. Human epidermoid carcinoma SiHa, established from fragments of a primary tissue sample of an undifferentiated squamous carcinoma of the cervix, was maintained in modified MEM supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and 1× nonessential amino acids. Both cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown to approximately 85–90% of confluence in a humidified atmosphere of 5% CO2 at 37°C.

RA (Sigma, St. Louis, MO) was added to the medium from a stock solution of 10−2 M in DMSO to a final concentration of 10−6 M or other, as described. Cells treated with the same volume of DMSO were used as a control in all of the experiments performed.

Recombinant IFN-α2b (INTERON A; 2 × 106 U/mg of protein; Shering Corp.) was added to the medium from a stock solution of 10² U/ml.

Measurement of Cell Proliferation. Cells were cultured in duplicate in 35-mm dishes starting at an initial density of 3 × 10⁵ cells/dish. After 24 h from seeding, appropriate dilutions of RA and/or IFN-α2b were added to the medium, and the cells were grown for 6 days in the absence or presence of supplements and DMSO, used as a control for RA. DMSO (0.1%) did not affect proliferation of the cells. To be counted, cells were detached after a previous washing with 100 mL EDTA followed by a 10-min exposure at 37°C to a solution of 0.1% Trypsin-2 mL EDTA in PBS (pH 7.2) and suspended repeatedly to give a single-cell suspension. Cells were counted using a hemocytometer. Cell mortality was evaluated by the trypan blue dye exclusion method.

RNA isolation and Northern Blot Analysis. Total cellular RNA was isolated and purified by the guanidine thiocyanate–cesium chloride method (42), quantitated by absorbance at 260 nm, analyzed by agarose formaldehyde gel electrophoresis, and transferred to Hybond-N membranes. The membranes were then prehybridized at 65°C for 1 h in Church's buffer [0.5 M NaPi (pH 6.8); 7% SDS] with 100 mg/mL calf thymus DNA and hybridized for 24 h at 65°C with 1.5 × 10⁶ dpm/mL random-primed ³²P-labelled human 2-5A synthetase cDNA (1.32-kb EcoR I insert subcloned in pBR322 cells) DNA (Ref. 24), mouse TGase II, TG700 (a generous gift of Dr. Peter J. A. Davies, Department of Pharmacology, University of Texas, Houston, TX), and human GAPDH. The blots were sequentially washed for 10–15 min each at 65°C in 1 × SSC-0.1%SDS, 1 × SSC-0.1%SDS, and 0.1 × SSC-0.1%SDS and exposed at 80°C to

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X-ray film. Autoradiographs were quantitated by densitometric analysis using an UltraScan XL densitometer (Pharmacia LKB).

Preparation of Cell Extracts. Cells (2–3 × 10⁶) were washed twice in ice-cold PBS-EDTA, scraped, and then collected by centrifugation at 1500 rpm at 4°C for 10 min. They were washed again twice in PBS, and the postmitochondrial supernatant fraction was prepared from packed cells lysed in 1.5 volumes of homogenization buffer [10 mM Tris-HCl (pH 7.5), 7 mM mercaptoethanol, 10 mM KCl, 1.5 mM MgOAc₂, and 0.5% NP40] at 4°C for 30 min and centrifuged at 10,000 × g for 10 min. The supernatant (S-10) was either assayed immediately or stored in aliquots at −80°C. Protein concentration was determined by the Bio-Rad DC protein assay.

TGase Assay. Cells grown in 60-mm dishes were washed in ice-cold PBS containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by three freeze and thaw cycles. The homogenate was centrifuged at 105,000 × g, yielding the particulate and soluble fractions. TGase assays were performed on the total homogenate to determine total TGase activity and on the particulate and soluble fractions to determine the activity of the type I and type II TGases, respectively. TGase activity was measured by determining the incorporation of [3H]putrescine (16.2 Ci/mmol, Dupont New England Nuclear) into casein hydrolysate (Sigma) as described (43) and was expressed as dpm of [3H]putrescine incorporated in 1 h of total cellular protein.

Western Blot Analysis. Whole-cell lysates from SiHa cells were prepared in lysis buffer [0.5% NP40, 10% glycerol, 50 mM Tris-HCl (pH 8), 0.1 mM EDTA, 150 mM NaCl, and 1 mM DTT; 0.4 mM phenylmethylsulfonyl fluoride, 3 µM/ml aprotinin, 1 µM/ml leupeptin, 0.5 µg/ml pepstatin, 50 mM NaF, and 1 mM sodium orthovanadate] were freshly added to the buffer before each use, electrophoresed on a 7% SDS-polyacrylamide gel, and transferred to nitrocellulose for 60 min at 100 V with a Bio-Rad transblot. Western blot detection was performed with guinea pig polyclonal antibody against TGase II (a generous gift of Dr. S. Beninati, Department of Biology, Faculty of Science, University of Rome "Tor Vergata") and developed with reagents for enhanced chemiluminescence (Amersham). Protein concentration was determined by the Bio-Rad protein assay.

DNA Fragmentation Analysis. DNA fragmentation was analyzed by a modification of the method reported previously (44). Fragmented DNA normalized on cell number was electrophoresed in a 1.5% agarose gel in 1× TBE buffer [0.05 M Tris base, 0.05 M boric acid, and 1 mM EDTA (pH 8.0)] and visualized by ethidium bromide staining.

Morphological Analysis. For Hoechst 33258 fluorescence staining, detached cells were first collected by centrifugation and resuspended in PBS. An aliquot of these (2 × 10⁶ in 40 ml) was seeded on pollysine-coated coverslips for 15 min and fixed with 3% formaldehyde in PBS (pH 7.4) for 10 min at room temperature. The cells adhering to the substrate were fixed and processed using the same methods. After washing in the same buffer, the cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Finally, after washing, all samples were stained with Hoechst dye and then mounted with glycerol-PBS (2:1) and observed with a Nikon Microphot fluorescence microscope. Quantitative evaluation of apoptotic cells by Hoechst staining (45) was performed by counting at least 500 cells at high magnification (×500). These analyses were carried out: (a) in the entire cell population (detaching adhering cells by using a policeman); and (b) by counting detached cells and adhering cells separately (41). Statistical analysis. The comparison of the results obtained in the same cell line by different treatments versus control cells was performed by using the Student's t test. A P value <0.05 was considered significant.

RNase Protection Analysis. A 400-bp Smal restriction fragment was derived from the human IRF-1 cDNA clone pUC28-8 subcloned in pBS/ KGI vector (Promega, Madison, WI) and used as a template to generate a 32P-labeled antisense riboprobe following transcription by T7 RNA polyme rase using EcoRI linearized template. A 316-bp SacI-BamHI restriction fragment of pTRI-PGAP/H human cDNA clone (Ambion, Inc., Austin, TX) was used as a template to generate a 32P-labeled antisense riboprobe following transcription by T3 RNA polymerase using HindIII linearized template. It was included in each reaction as internal control. Briefly, the 32P-labeled antisense riboprobe (3 × 10⁶ cpm) were hybridized for 12–16 h with 50 µg of total cellular RNA at 55°C. The samples were then digested with RNase A and RNase T1 (Boehringer Mannheim), extracted, and ethanol-prefi citated before being separated on 8% polyacrylamide gels containing 8 % urea. 32P-Labeled, sized markers were also run on the gels. The gels were then dried and exposed to X-ray film for 1–3 days before being density metrically analyzed.

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