INTERFERON EFFECTS ON MICROFILAMENT ORGANIZATION, CELLULAR FIBRONECTIN DISTRIBUTION, AND CELL MOTILITY IN HUMAN FIBROBLASTS

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ABSTRACT

We have shown previously (Pfeffer et al., 1979, *Exp. Cell Res.* **121**:111–120) that treatment of human fibroblasts, planted at a density of 2×10^3 cells/cm^2, with purified human fibroblast interferon (640 U/ml) for 3 d at 37°C decreases the overall rate of cell proliferation to 35–40% of the control value. In the present experiments we have characterized the phenotype of interferon-inhibited fibroblasts. The mean volume of trypsinized, interferon-treated cells was increased 31% above that of control cells. The interferon-treated population was much more heterogeneous than the control population with respect to volume, and there was a considerable overlap in the volume distributions of the two populations. The cell surface area was, on the average, increased 65% after interferon treatment. More than 80% of the treated cells had enlarged nuclei, many of which were lobed, and the fraction of binucleated cells was increased fivefold. After interferon treatment, over 40% of the cells showed large actin-containing fibers in the form of multiple parallel arrays. Fewer than 5% of the control cells contained such large actin fibers. The number of actin fibers of all sizes was tripled in the treated fibroblasts on a per cell basis and, calculated per unit surface area of the cells, the number was increased 82%. In contrast, 10-nm filaments and microtubules did not appear to be increased in number per unit surface area of the cells. The increases per cell in the abundance of these structures were directly related to increased cell size. After interferon treatment, fibronectin was distributed in arrays of long filaments covering most portions of the cell surface. Interferon treatment markedly decreased the rate of cell locomotion as well as membrane ruffling and saltatory movements of intracellular granules.

Although originally characterized as antiviral agents, interferons have been shown to have multiple effects on cells (6, 18, 20). An outstanding property of interferons is their ability to inhibit the proliferation of both normal and transformed cells (2, 9, 14, 19, 23, 24, 37, 40). Time-lapse cinemicrography has shown that interferon treatment causes a progressive increase in the intermitotic interval in mouse EMT6 cells (9) and in human fibroblasts (40). Furthermore, over the course of several days after the beginning of treatment, an increasing proportion of cells fails to divide again. Thus, the full effect of interferon on cell proliferation in a population is expressed only over the course of several cell generations, as the response of the cells in the population is heterogeneous.

Although the rates of DNA, RNA, and protein

synthesis are somewhat depressed in interferon-treated cells, the cells increase in mass (40). After treatment of virus-transformed human RSa cells with leukocyte interferon for 4 d, the protein content of cells was increased approximately twofold (14). Treatment of human fibroblasts with fibroblast interferon for 3 d caused a 50% increase in protein content (40). As has been noted previously (40, 52), interferon-treated cells behave in several respects as senescent fibroblasts in vitro. Senescent fibroblasts also display a progressive decline in proliferative ability, marked by heterogeneity in the behavior of the population (1, 16, 17, 27, 32–35, 47–49). The progressive decline is accompanied by a reduction in the saturation density achieved by the monolayer, an increase in the mean cell size, and a greater heterogeneity of cell sizes in the population.

These observations raise the possibility that a common cellular response is evoked when the proliferation of cells becomes restricted either through interferon treatment or through the life-span mechanism which limits the proliferation of diploid fibroblasts in vitro. This adds to the importance of a full characterization of the structure and function of interferon-treated cells.

In the present study we have determined that treatment of human fibroblasts with human fibroblast interferon (640 U/ml) for 3 d results in increased mean cell size and volume. The enlarged and well-spread interferon-treated cells commonly have enlarged lobed nuclei, and the number of binucleated cells is increased. The actin-containing cytoplasmic fibers are markedly increased in size and number, and the cell surface fibronectin is arranged in arrays of long filaments covering the surface of interferon-treated cells. The motility of interferon-treated cells is markedly decreased. Preliminary reports of a part of this work have appeared previously (39, 42).

MATERIALS AND METHODS

Cell Cultures

Human diploid fibroblast strains FS-4 (53) and ME (38) between the 10th–20th passage levels in culture were grown in monolayers in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Interferon Preparations and Assay

Human fibroblast interferon, purified to a sp act of 2×10^7 U/mg protein, was generously provided by Drs. W. A. Carter and J. S. Horoszewicz of Roswell Park Memorial Institute. Purification had been carried out by chromatography on concanavalin A agarose in tandem with phenyl Sepharose columns. In addition, one lot of human fibroblast interferon was obtained from Dr. J. Vilček (New York University). This had been purified by carboxymethyl-Sephadex chromatography to a sp act of 5×10^5 U/mg protein. Interferon was assayed by a semimicro-titration procedure (21) performed on monolayers of ME fibroblasts inoculated with vesicular stomatitis virus. Interferon titers, based on inhibition of viral cytopathic effects, are expressed in terms of U/ml, using NIH reference standard 69/19 for comparison.

Cell Volume Analysis

Human fibroblasts were seeded onto 75-cm^2 flasks at a density of 2×10^3 cells/cm^2 in MEM supplemented with 10% FCS. 1 d after planting cultures were re-fed with MEM containing 10% FCS with or without human interferon, 640 U/ml. After incubation at 37°C for 3 d, the control and interferon-treated cells were removed from the substrate by incubation for 5–10 min at 37°C in 1 ml of 0.1% *N*-acetyltrypsin, 1.2% ethylenediamine tetraacetate in phosphate-buffered saline (PBS). Cells dislodged from the flasks were resuspended in the trypsin solution and transferred to tubes containing 4 ml of 4% glutaraldehyde in PBS chilled in ice. Cell volumes were determined electronically using a Coulter Channelyzer model H4 (Coulter Electronics Inc., Hialeah, Fla.). The cell volume analysis was kindly performed by Dr. R. Zucker (Papanicolaou Cancer Research Institute, Miami, Fla.).

Cell Surface Area

Human fibroblasts were planted at a density of 2×10^3 cells/cm^2 in MEM supplemented with 10% FCS into 35-mm petri dishes which contained three to five sterile round 12-mm cover glasses. 1 d after planting, cell cultures were re-fed with MEM containing 10% FCS with or without human interferon, 640 U/ml. 3 d after re-feeding, coverslips were removed from dishes and fixed with 3.7% formaldehyde in PBS. Photomicrographs were made in a Zeiss model III RS phase-contrast microscope to which was attached a 35-mm camera with an automatic exposure meter. As a standard of reference, photomicrographs were taken of a calibrated grid. The negatives were developed and enlarged 5.7 times and printed on high-contrast paper. The areas encompassed by individual fibroblasts were cut out and weighed. Photographs of a known area of the reference grid were taken at × 127, × 200, and × 438. The known area was cut out and weighed to determine the factor by which weight values for cells could be translated into absolute areas.

Indirect Immunofluorescence Microscopy

Coverslip cultures of human fibroblasts were prepared as described above. Actin-containing microfilament bundles were localized by incubation of formaldehyde-fixed and acetone-treated cells with deoxyribonuclease I (DNase I) in combination with antiserum to DNase I. The specificity of this procedure for visualization of actin fibers has been documented previously (56). 10-nm filaments were visualized by means of antiserum (a generous gift of Dr. R. D. Goldman) directed against the 53,000- and 54,000-dalton protein subunits of this cytoplasmic fiber (50). Cytoplasmic microtubules were localized by using antiserum prepared against purified 6S calf brain tubulin. The procedures for tubulin isolation and characterization of the rabbit antiserum

to this protein have been previously described (54). After application of the respective antisera, coverslips were processed for indirect immunofluorescence as previously described (57). Stained cells were examined in a Zeiss photomicroscope III RS equipped with epifluorescence. The procedure for examination of cells for cellular fibronectin was similar, except that the acetone extraction step was omitted. The antiserum to fibronectin was obtained from A. B. Chen and M. W. Mosesson through the courtesy of Dr. L. B. Chen. This antiserum has been shown to react specifically with cellular fibronectin (K. Burridge, quoted in reference 7).

Cell Locomotion

Human fibroblasts (ME cells) were planted into Falcon flasks (25 cm^2; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) which were equilibrated with 5% CO_2 in air. After re-feeding with MEM containing 15% FCS, the control and interferon-treated (640 U/ml) cultures were photographed at 2-min intervals under phase-contrast optics with a × 10 objective. Developed films were projected and the movement of individual cells was traced onto paper within consecutive 12-h intervals. The rate of locomotion was then calculated, as described in Table II.

RESULTS

General Morphology of Interferon-treated Human Fibroblasts

After 3 d of treatment of human fibroblasts with fibroblast interferon, 640 U/ml, the overall rate of cell proliferation is decreased to 35–40% of the control value (40). At this time, over one-half of the treated cells appear both enlarged and flattened out. Frequently, the nuclei are abnormal in shape and numerous binucleated cells are present. Time-lapse cinemicrography has shown that most of the binucleated cells arise through aberrant mitosis. As illustrated in Fig. 1, phase-contrast microscopy reveals large stress fibers in the cytoplasm of interferon-treated cells. Such fibers are rarely observed in control cells. However, a subpopulation of cells in the interferon-treated cultures is indistinguishable from control cells in general characteristics.

Cell Volume

The volume distribution of interferon-treated and control cells was determined after removal from culture flasks with trypsin and fixation in glutaraldehyde. The interferon-treated cell population was much more heterogeneous (coefficient of variation, c.v. = 21.5) than the control population (c.v. = 11.5). Furthermore, the mean volume of interferon-treated cells was 3.97×10^3 μm^3, whereas that of control cells was 3.03×10^3 μm^3, which represents a 31% increase. However, there is a considerable overlap in the cell distributions of interferon-treated and control cells. Because these measurements were made after dispersion and suspension of cells from monolayer cultures, the estimates obtained do not give the actual volumes of cells in culture.

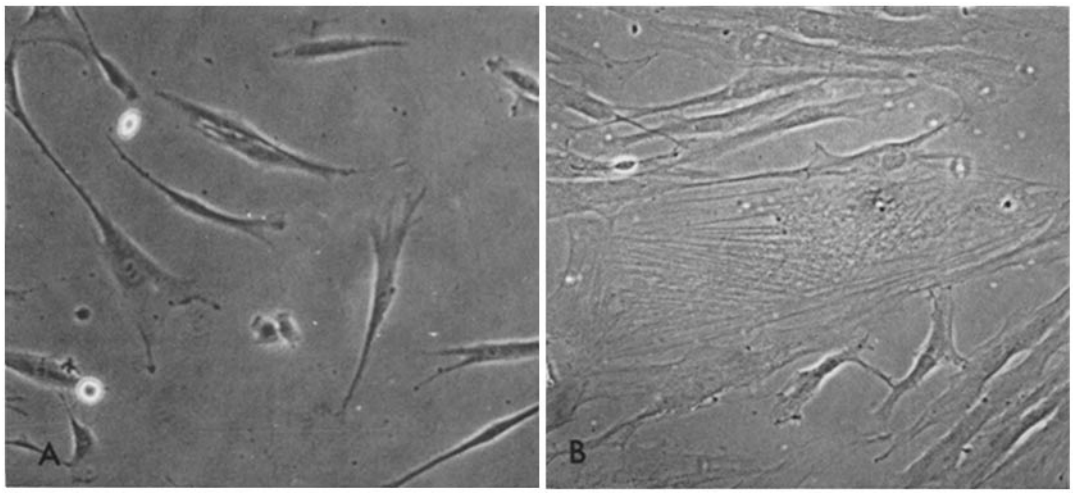

FIGURE 1 Effect of interferon treatment on the morphology of human fibroblasts in culture. Cells of strain ME human skin fibroblasts were grown on coverslips and processed 3 d after the beginning of interferon treatment (640 U/ml), as described in Materials and Methods. Phase-contrast micrographs of control (*A*) and interferon-treated (*B*) cells were taken at the same magnification. Note the increased cell size and appearance of large stress fibers in Fig. 1 *B*. Cells of strain FS-4 human newborn foreskin fibroblasts showed similar characteristics after treatment with interferon. × 375.

Cell Surface Area

Fig. 2 shows frequency distributions of surface areas occupied by interferon-treated and control cells. Only 5.5% of the control cells show values over 5,000 μm^2, whereas 18% of interferon-treated cells have areas in excess of 7,500 μm^2. The mean attachment-surface area of control cells is 2,540 ± 160 μm^2, whereas for treated cells it is 4,200 ± 330 μm^2, which represents a 65% increase. It can be computed that human fibroblasts that have been treated with interferon for 3 d occupy a similar portion of the available growth surface (52%) as control cells (60%). Therefore, inhibition of cell proliferation by interferon is probably not caused by altered sensitivity of interferon-treated cells to density-dependent inhibition as was previously suggested (26).

Nuclear Characteristics

A correlation between cell volume and nuclear size has been demonstrated in several mammalian cell systems (12, 13, 33, 44). Table I summarizes frequency distributions of nuclear characteristics in interferon-treated and control human fibroblasts. Over 80% of control cells but only ~20% of interferon-treated fibroblasts have relatively small, oval nuclei. Many of the enlarged nuclei in interferon-treated cells are polymorphic and show varying degrees of lobation. In addition, there is a fivefold increase in binucleated fibroblasts after interferon treatment.

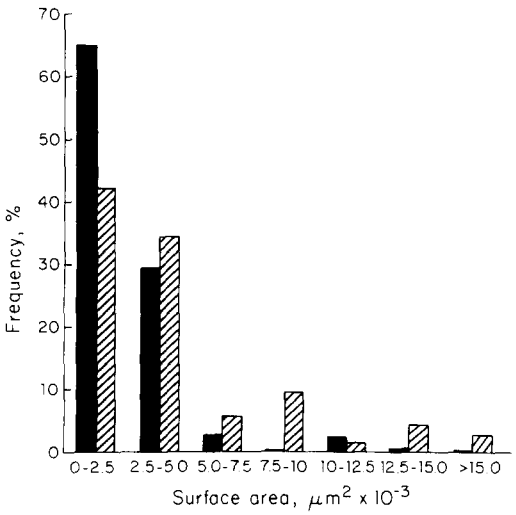

FIGURE 2 Surface area distribution of control and interferon-treated human fibroblasts. ME cells were grown on cover glasses and processed and photographed 3 d after the beginning of interferon treatment (640 U/ml), as described in Materials and Methods. Between 150 and 200 cells of each culture were photographed through a × 16 phase-contrast objective (total magnification: × 200), and 8 × 11 prints made. The cut-out areas representing individual cells were weighed, and the weights were converted to surface areas (2,500 μm^2 was equivalent to 0.27 g under the conditions used).

TABLE I

Nuclear Characteristics of Control and Interferon-treated Human Fibroblasts

	Frequency*			
	Binucleate	Large	Intermediate	Normal
	%			
Control	1.7	4.3	11.2	82.8
Interferon	8.4	33.9	38.9	18.8

* ME cells were grown on cover glasses and examined through a × 63 phase-contrast objective (total magnification: × 900) 3 d after beginning of treatment. 340 control cells and 417 interferon-treated (640 U/ml) cells were scored for the presence of two nuclei and for nuclear size.

Cytoskeletal Components

Fig. 3 illustrates the distribution of actin-containing microfilaments in control and interferon-treated cells as revealed by immunofluorescence staining. Control cells show a few prominent fibers scattered in the cytoplasm (Fig. 3*A*). Fewer than 5% of the control cells have actin-containing cables 4,000–8,000 Å in width. In contrast, over 40% of interferon-treated fibroblasts exhibit such large and prominent actin-containing fibers throughout the cells, as visualized by immunofluorescence microscopy (Fig. 3*B*). The treated cells contain multiple arrays of parallel fibers, many of which span the length of the cell. By adjusting the depth of focus, it was determined that most of these fibers were located in the plane adjacent to the attachment surface of the cell.

We also enumerated actin-containing cables in randomly selected control and interferon-treated cells, with ~100 cells in each sample. The number of actin fibers of all sizes was increased ~200% on a per cell basis in interferon-treated cells as compared to control cells. As reported above, interferon treatment caused a 65% increase in the surface area of cells. Therefore, we estimate that the number of actin fibers, calculated per unit surface

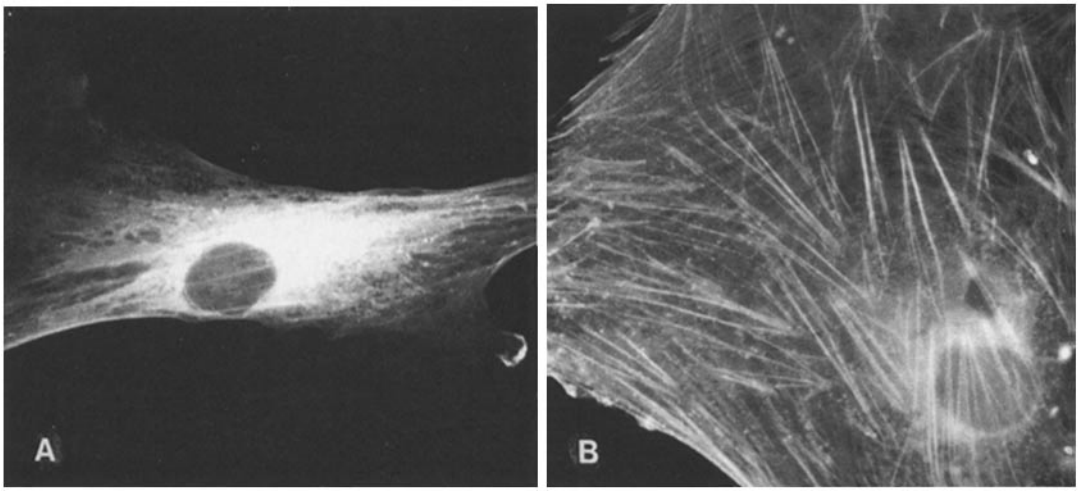

FIGURE 3 Effect of interferon treatment on the distribution of actin-containing microfilaments in human fibroblasts. Cells were grown on cover glasses and stained for intracellular actin by the indirect immunofluorescence technique (see Materials and Methods). Panel *A*: control ME cells; panel *B*: interferon-treated (640 U/ml, 3 d) ME cells. Note the appearance of numerous large actin-containing fibers in interferon-treated cells. Many bundles appear to span the entire length of the fibroblast. Similar observations were made with FS-4 cells. × 900.

area, is increased 82% in interferon-treated fibroblasts.

The patterns of immunofluorescence staining for microtubules and 10-nm filaments in interferon-treated cells were similar to those in control cells. These cytoskeletal components also were more abundant on a per cell basis in interferon-treated cultures. However, in contrast to the findings with actin fibers, there was little difference in the number of either 10-nm filaments or microtubules per unit cell surface area in interferon-treated as compared to control cells.

Distribution of Fibronectin on the Cell Surface

Corresponding to the striking changes in the organization of intracellular microfilament bundles, interferon treatment of human fibroblasts markedly alters the distribution of cell surface fibronectin. Fibronectin is distributed in control cells as a network of fibers found mainly at regions of cell-to-cell contact (Fig. 4 *A* and *B*). After interferon treatment, cellular fibronectin is distributed in arrays of long filaments covering most portions of the cell surface (Fig. 4 *C* and *D*). The cell surface fibronectin appears to be aligned with the actin-containing microfilament bundles in the cytoplasm of interferon-treated fibroblasts.

Cell Locomotion, Membrane Ruffling, and Intracellular Movement

Time-lapse cinemicrography has revealed that the motility and intracellular movements of ME cells are reduced after interferon treatment (640 U/ml). The locomotion of randomly selected human fibroblasts in control and interferon-treated cultures was followed during consecutive 12-h intervals. As shown in Table II, the mean rates of cell locomotion in control cultures were within 0.1070–0.1417 μm/min throughout the course of the 96-h period of experiments. The rate of cell locomotion in interferon-treated cultures was similar to control values during the first 24 h of treatment. However, for the period from 24 to 36 h after beginning of interferon treatment, the rate decreased to 44% of the overall mean control value and it leveled off at ~20% of the control value 60–72 h after the beginning of treatment. Examination of high resolution cinemicrographs has revealed that the saltatory movements of intracellular granules and membrane ruffling are markedly reduced in interferon-treated cells. Additional data are necessary to provide quantitative measurements of these observations.

As described above, interferon treatment of human fibroblasts results in an increased organization and abundance of actin-containing micro-

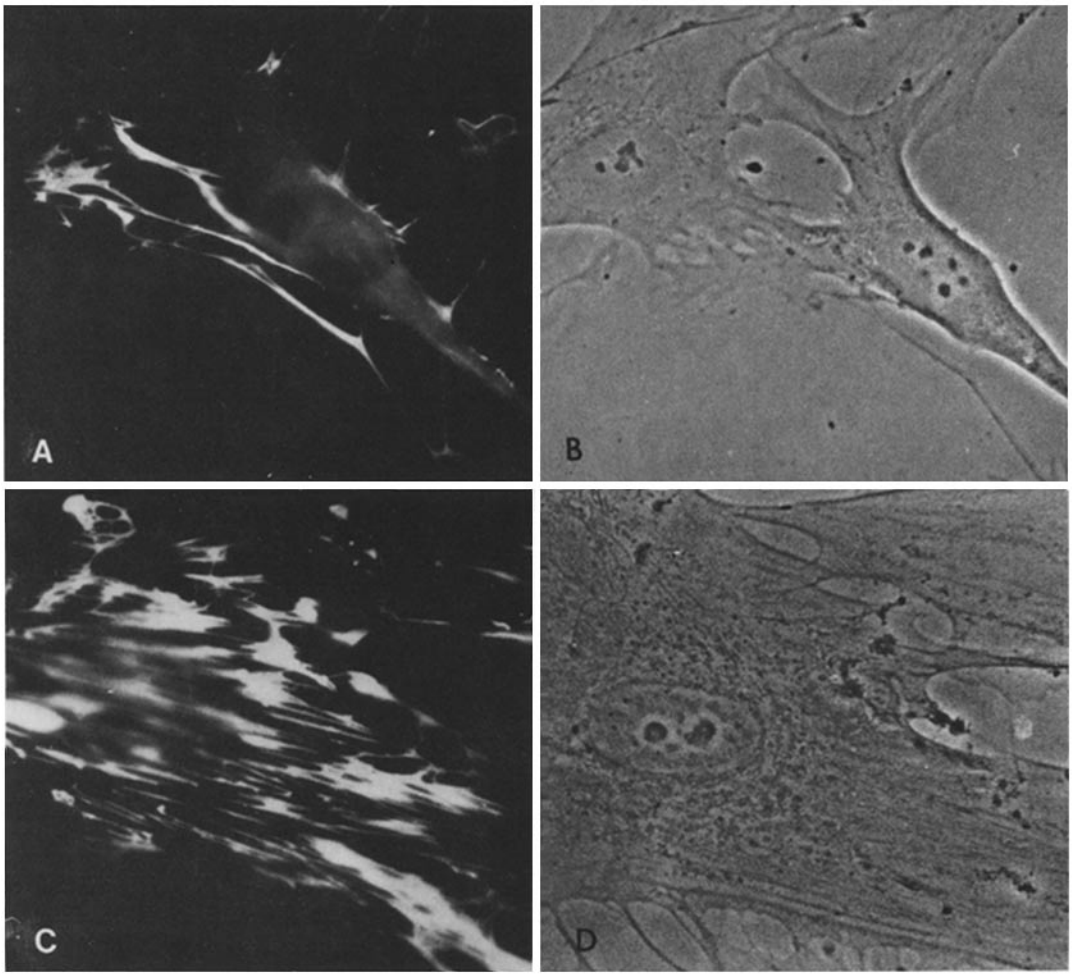

FIGURE 4 Effects of interferon treatment on the extracellular distribution of fibronectin in human fibroblasts. Control and interferon-treated (640 U/ml, 3 d) ME cells were stained for fibronectin (see Materials and Methods). Cells were examined under epifluorescence and by phase-contrast optics using a × 63 phase-contrast objective. Control cells: immunofluorescence (*A*), phase-contrast (*B*); interferon-treated cells: immunofluorescence (*C*), phase-contrast (*D*). Note that in control cells, fibronectin is distributed in pericellular areas, particularly in regions of cell-to-cell contact. In interferon-treated cells, fibronectin spans the entire surface of the cell in the form of filamentous structures. × 900.

filament bundles. Thus, the decreased motile activity observed after interferon treatment, involving cell locomotion, membrane ruffling, and saltatory movements, may be related to increased organization of microfilaments in interferon-treated fibroblasts.

DISCUSSION

We have demonstrated that the inhibition of proliferation of cultured human fibroblasts by human fibroblast interferon is associated with several outstanding phenotypic changes in cells, which, taken together, appear to constitute a coordinated response of the cells to the action of interferon. This response is characterized by an enlargement of cells, the presence of extraordinarily large actin-containing fibers in the cytoplasm and long filamentous arrays of fibronectin on the cell surface, and decreased cell locomotion and intracellular movements. Experiments carried out mainly in HeLa cells (human epithelioid carcinoma line) show that the response to interferon action also entails increased rigidity of the plasma membrane (41) and impairment of lateral movement of cell

TABLE II

Locomotion of Control and Interferon-treated Human Fibroblasts

Time	Cell locomotion		
	Control	Interferon-treated (640 U/ml)	% of Control*
h	*μm/min ± SEM*	*μm/min ± SEM*	
0–12	0.1251 ± 0.0069 (44)	0.1332 ± 0.0144 (35)	105
12–24	0.1310 ± 0.0095 (48)	0.1149 ± 0.0098 (35)	90.6
24–36	0.1259 ± 0.0073 (55)	0.0556 ± 0.0054 (31)	43.8
36–48	0.1290 ± 0.0069 (56)	0.0549 ± 0.0076 (34)	43.3
48–60	0.1290 ± 0.0077 (62)	0.0375 ± 0.0042 (39)	29.6
60–72	0.1417 ± 0.0110 (43)	0.0273 ± 0.0019 (35)	19.3
72–84	0.1257 ± 0.0083 (42)	0.0247 ± 0.0027 (33)	19.4
84–96	0.1070 ± 0.0073 (40)	0.0238 ± 0.0022 (37)	18.8

The experimental conditions are given in Materials and Methods. The paths of movement of individual cells in cinemicrographs were traced onto paper with the aid of a stop motion projector. The paths were measured with a distance tracking device, and the measurements were divided by the total magnification. Rate of cell movement was expressed in μm/min.

Numbers in parentheses are the cell numbers.

* A mean control value of 0.1268 μm/min, based on all measurements during the entire period of observation, was used in computing % of control values. The results recorded are based on four separate experiments.

surface components such as receptors for concanavalin A (41) and cholera toxin.[1]

Even after 3 d of treatment of human fibroblasts with interferon at a concentration (640 U/ml) at which essentially maximal inhibition in the overall rate of cell proliferation is observed (40), some cells are still capable of division in the presence of interferon and appear morphologically unaltered. There is much evidence that in the original population of the diploid fibroblasts there are present subpopulations of cells differing in their proliferative potential (16, 27, 48, 49). Cloning of mass cultures of skin fibroblasts has revealed a bimodal distribution of proliferation rates and numbers of population doublings among the clonal populations. The clones which are capable of few cell divisions and have been described as "terminally differentiating" consist of cells which are larger and more epithelioid than those in clones regarded as constituting the "proliferative pool" (27). The "terminally differentiating" cells have been noted to contain an abundance of filamentous structures (27). It will be of interest to determine whether the subpopulation of fibroblasts which already has low proliferative potential is particularly sensitive to the antiproliferative action of interferon, or whether the heterogeneous response of fibroblasts to interferon has some other basis.

In several studies evidence has been obtained of an inverse relationship between the rate of proliferation and cell size in cultured human fibroblasts (1, 8, 17, 33–35, 46). An increased cell size is associated not only with the process of in vitro aging of diploid fibroblasts and with interferon treatment; a similar shift can also be obtained by reducing serum concentration, decreasing incubation temperature, and inhibiting DNA synthesis with hydroxyurea (35). It thus appears that increased cell size is a common response to inhibition of cell proliferation by means which do not cause degenerative changes in cells. Because of the increased size of interferon-treated cells, such cells make contact at a lower population density than untreated fibroblasts. This is likely to accentuate further the inhibition of proliferation of the treated cells.

Of particular interest is the observation that the response of cells to the antiproliferative action of interferon entails an increase in the organization of the cytoplasmic actin-containing microfilaments and an altered distribution of the surface protein, fibronectin. These changes are opposite to those commonly associated with the unregulated proliferation of transformed cells. There is extensive evidence that the arrangement of actin-containing microfilaments in cables is lost or much reduced in cells transformed by simian virus 40 (SV40) or Rous sarcoma virus (RSV) (11, 28–31,

[1] Pfeffer, L. M., E. Wang, and I. Tamm. Interferon inhibits the redistribution of cell surface receptors for concanavalin A. Manuscript submitted for publication.

43, 55, 57, 58). It has been reported that, after transformation of chick embryo fibroblasts with RSV, actin is less extensively polymerized (44), and the amount of actin associated with membranes is decreased by 30–50% (60). Moreover, the disappearance and reappearance of actin-containing microfilament bundles both in transformed and normal mouse and rat cells have been reported to be related to the rate of cell proliferation (15).

The amount of fibronectin on the cell surface generally is reduced upon oncogenic transformation, and the extent of loss correlates with an increase in tumorigenicity (7, 22). The addition of purified fibronectin restores the normal morphology in transformed cells (3, 62). Indeed, the actin-containing microfilament bundles are restored to a normal pattern in transformed cells in fibronectin-replenished cultures (3, 61).

It is thus clear that the microfilaments and fibronectin are cellular elements that are subject to profound changes as the cell expresses its proliferation-related phenotype. Interferon-induced inhibition of cell proliferation is associated with changes that typify one end of the phenotypic spectrum, whereas changes associated with tumor virus-induced uncontrolled cell proliferation typify the other end. It is possible, if not probable, that these changes are not specifically and directly caused by interferon and oncogenic viruses, respectively, but that they instead represent two outstanding manifestations of complex coordinated responses of cells to agents that affect their proliferative potential.

There is evidence that the proliferation of cells is controlled mainly in the G_0–G_1 phase of the cell cycle (4, 36). Oncogenic viruses enable cells to traverse G_1 into S under conditions (high cell density, low serum concentration) under which untransformed cells fail to do so (5, 10, 51, 59). Human fibroblasts whose proliferation in vitro is decreasing through the life-span mechanism accumulate in G_1 (46). Experiments with serum-depleted fibroblasts which were stimulated by serum to proliferate synchronously have indicated that interferon inhibits an early G_1 process (25). Observations by flow cytometry also suggest that interferon-treated fibroblasts behave as if a G_1 process were impeded, as there is an increase in the proportion of G_1 phase cells (52). Further studies are needed to relate the contrasting coordinated responses of cells to oncogenic viruses, on the one hand, and to the life-span mechanism and interferon, on the other, to specific control mechanisms which govern cell proliferation.

At present there is relatively little information about the phenotypic changes in human fibroblasts whose proliferation is terminated by the life-span mechanism. The available information, which includes the finding that late-passage (52) human fibroblasts are less motile than earlier-passage (28) cells (1), appears sufficient to raise the possibility that a common response pathway may operate in fibroblasts whose proliferation is restricted by either the life-span mechanism or interferon. We propose that this response pathway involves coordinated changes in the plasma membrane and the cytoskeleton. Studies of interferon action suggest that changes in membrane fluidity, altered distribution of cell surface fibronectin, and formation of very large actin-containing microfilament bundles may in themselves interfere with cytokinesis as well as cell locomotion.

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