Release of Surface-Associated Phosphoesterases from HeLa Cells by Ficin[1] (36059)

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Renewed interest in the plasma membrane has produced information about basic architecture and physiological function of this organelle. Some recent findings were derived from studies of various agents, which specifically affect membrane properties of cells in culture; some were revealed in the course of a search for differences in surface characteristics between neoplastic and normal cells. Abercrombie (1), Stoker (2), and Ambrose (3) provided evidence that the electrophoretic mobility, cell movement, and contact inhibition phenomena should be included in the list of gross alterations distinguishing cells which have undergone neoplastic transformation from their normal counterparts. Several reports (4–6) pointed to alterations in the amino sugar composition of the cell surface as a consequence of viral transformation.

Among agents capable of altering the surface properties of cells in culture are various hydrolytic enzymes including *N*-acetylneuraminidase, trypsin, and proteolytic enzymes of plant origin such as papain and ficin. Since most of the surface charge in cultured cells is attributed to the carboxyl groups of *N*-acetylneuraminic acid (7), it is not surprising that the removal of glucosidically linked sialic acid with *N*-acetylneuraminidase reduces their electrokinetic mobility (8). Contrarily, Kraemer (9) considered that the changes in electrokinetic acitvity are due to a topological alteration of the sialic acid moieties rather than the charge density of the terminal residues. Significantly, the selective agglutination of malignant cells by wheat germ lipase preparations (10) is potentiated by growing the cells in the presence of prednisolone and is abolished by treating the cells with *N*-acetylneuraminidase (11).

Trypsin has been used for many years for disaggregating tissue in preparation for culturing cells and is widely employed for detaching cells from glass. In general, cells seem to tolerate such treatment without loss of viability. However, there is considerable evidence (12) that they lose macromolecular components after being treated with trypsin. Less is known about the effect of papain and ficin. Although, like trypsin, papain releases a sialoglycopeptide from the cell surface (13), there are no reports on the loss of intracellular material from cells treated with this or with the related enzymes.

The purpose of this investigation was to assess the overall effect of ficin on cultured cells. Particular emphasis was placed on the release of enzymes known to be associated with the cell membrane and on cell survival as a consequence of ficin treatment. Since membrane properties of cultured cells are affected by glucocorticoids (14, 15), an attempt was also made to study the effects of prednisolone during growth.

Materials and Methods. Two different HeLa S3 strains: S3G (courtesy of Dr. M. Griffin; Oklahoma Research Foundation) and S3K (courtesy of Dr Kazuto Kajiwara; University of Wisconsin) were used. The S3G cells have a low level of alkaline phosphatase which becomes elevated in response to glucocorticoids; the S3K strain has a high level of this enzyme which is suppressed if the culture is maintained in the presence of prednisolone (16). Both sublines were grown in water dilution bottles or in Bellco roller bottles (Bellco Glass Co., Vineland, NJ) in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum and with

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TABLE I. Effect of Ficin on Cloning Efficiency of Two HeLa Sublines.

Incubation medium	Cloning efficiency[a]			
	HeLa S3G		HeLa S3K	
	Control	Prednisolone	Control	Prednisolone
Eagle's MEM + 10% calf serum	77[b]	61	35	28
50 m*M* Tris, pH 7.2, in physiological saline	49	49	40	36
0.4% Ficin in Tris–saline, pH 7.2	61	59	54	58

[a] Cells pregrown either with or without prednisolone in Puck's medium with 10% calf serum. An inoculum of 100 cells/petri dish was used. No steroid was added following inoculation of cells for cloning.

[b] Means of number of colonies from three petri plates.

penicillin and streptomycin at concentrations of 100 units/ml. The cultures were monitored regularly for contamination with mycoplasma according to the method of Shedden and Cole (17) and were found to be negative.

Crude ficin (Sigma Chemical Company, St. Louis, MO) was dissolved in 0.85% NaCl solution containing 50 m*M* Tris at pH 7.2. The final concentration of ficin was 4 mg/ml, approximately equivalent to 1 unit of enzyme/ml. The cells grown on glass surface for 3 days were detached by scraping with perforated cellophane and were washed in Tris–saline. They were then centrifuged at 600*g* for 5 min. The supernate was removed and the pellet was suspended in ficin solution and incubated for 30 min intervals at 37°. Next the suspension was centrifuged as before at 600*g* for 5 min, the supernatant fluid and the pellet were separated and, if not used immediately, stored at −10° before use in various assays. Control cell suspensions containing no ficin were prepared in like manner.

The enzyme assays were carried out as follows: alkaline phosphatase was assayed as described earlier (18), but using 0.5 *M* Tris buffer, pH 10.1. The 5′-nucleotidase activity was determined by a modified method described by Amador and Wacker (19). Tris buffer (20 m*M*) was substituted for the barbital buffer and the high concentration of $MgSO_4$ was omitted from the reaction mixture. The (Na^+, K^+)-ATPase was assayed according to the method of Manitius *et al.* (20) and the inorganic pyrophosphatase as described by Melani and Farnararo (21). The activities of all phosphoesterases were expressed as millimicromoles of inorganic phosphate released per minute.

Protein was determined by the Oyama and Eagle modification (22) of the Lowry method. Cell cloning was carried out as described by Puck *et al.* (23). Cell viability was assayed by the dye exclusion method using an 0.5% solution of trypan blue in saline.

Results. As shown in Table I, prednisolone alone did not affect the cloning of HeLa cells. Moreover, the incubation of freshly harvested cells in ficin did not have any adverse effects on the cloning efficiency of either of

TABLE II. Dye Exclusion Assay in HeLa S3G Cells Exposed to Ficin.

Time of sampling (min)	% Stained	
	Control	Prednisolone
Before ficin addition	14.5[a]	8.0
After ficin addition 1	59.6	48.2
10	13.4	11.5
20	12.3	10.5
30	15.2	15.9
60	6.9	6.8
120	2.9	2.8
240[b]	37.3	31.8

[a] The cell suspensions incubated in ficin solution for the indicated length of time were mixed with an equal vol of 0.5% trypan blue, placed in a hemocytometer and counted immediately. From 500 to 1000 cells were counted for each time interval.

[b] Considerable amount of cell debris was noticeable at this point.

TABLE III. Release of Protein Containing Material from HeLa Cells Treated with Ficin.

Subline	Growth	Treatment	Protein[a]		
			Pellet (mg)	Supernate (mg)	% in supernate
HeLa S3G	Control	Buffer	31.6[b]	1.5	4.6
	Prednisolone	Buffer	35.6	1.2	3.4
	Control	Ficin	27.9	5.3	16.0
	Prednisolone	Ficin	34.1	3.6	10.0
HeLa S3K	Control	Buffer	29.7	1.9	6.3
	Prednisolone	Buffer	18.6	1.2	6.0
	Control	Ficin	24.8	10.9	30.0
	Prednisolone	Ficin	15.5	4.6	23.0

[a] The cells were grown for 3 days either in the presence or in the absence of prednisolone (0.5 μg/ml), scraped with perforated cellophane, and treated with ficin as described in text.
[b] Means from two determinations.

the two sublines. Table I discloses a moderate increase in the number of colonies derived from cell suspensions that had been treated with this enzyme. Only in the case of the S3G cells incubated in the complete growth medium did the cloning efficiency exceed that obtained with ficin-treated cells. The apparent increase in the number of clones possibly reflected a better dispersal by the enzyme of cell clumps that otherwise would have given rise to single colonies. For this reason an attempt was made to confirm the cell cloning experiments by the dye exclusion method. Table II shows that a transient increase in permeability occurred almost instantly after addition of ficin to the cell suspensions resulting in about 50% of the cells taking up the dye. When the incubation in ficin solution was continued, the damage to the membrane seemed to be repaired. After 2 hr, the evaluation of the finin effect became unreliable because of an increasing amount of debris due to apparent cell disintegration.

The removal of cellular material was followed by measuring the amount of protein shed by the ficin-treated cells. The results are shown in Table III. The incubation of cells in buffer alone caused a loss of about 3 to 6% of total protein. Addition of ficin increased the protein leakage to about 16% for the S3G strain and to 30% for the S3K strain. With both strains, treatment during growth with prednisolone slightly decreased the extent of protein loss.

The effects of ficin on the release of four membrane-associated esterases are summarized in Table IV. As expected (16), there was a prednisolone-induced elevation of alkaline phosphatase in the S3G strain and a suppression in the S3K strain, while other phosphoesterases responded similarly but less consistently to this steroid. After treatment with ficin, variable amounts of activities of these enzymes were detected in the incubation medium. Noteworthily, a considerable amount of activity could also be demonstrated after incubation in the buffer alone, and generally ficin treatment merely increased the shedding of these enzymes. This was particularly noticeable in the case of pyrophosphatase S3G strain) and Na^+-, K^+-ATPase (both strains). As far as the steroid effect is concerned, the relative extent of shedding of phosphoesterases did not follow a uniform pattern. In the S3G strain prednisolone-treated cells released less enzyme when incubated in the buffer alone. However, if they were treated with ficin the relative amount of phosphoesterase activity released into the medium was similar in both steroid-treated and control cells. In the S3K strain, steroid-treated cells incubated in buffer released less inorganic pyrophosphatase Na^+-, K^+-ATPase, and 5′-nucleotidase than the controls but the shedding of alkaline phosphatase was somewhat increased in the steroid-treated cells. When the S3K cells were treated with ficin, the fraction of total

TABLE IV. Effects of Ficin on the Release of Four Cell Surface Associated Phosphoesterases.[a]

Enzyme		HeLa S3G Strain				HeLa S3K Strain			
		Control; incubated in:		Prednisolone; incubated in:		Control; incubated in:		Prednisolone; incubated in:	
		Buffer	Ficin	Buffer	Ficin	Buffer	Ficin	Buffer	Ficin
Alkaline phosphatase	Total[b]	4.3	6.2	10.3	10.6	197.4	290.4	50.6	34.5
	SN[c]	0.3	5.3	0.4	9.3	16.4	79.4	3.3	25.4
	% in SN[d]	7.0	85.5	3.9	87.7	8.3	27.3	6.5	73.6
Inorganic pyrophosphatase	Total	0.6	0.6	1.0	1.2	10.6	8.0	1.7	1.4
	SN	0.2	0.3	0.2	0.6	0.5	2.2	0.1	0.4
	% in SN	33.3	50.0	20.0	50.0	4.7	27.5	5.9	28.6
5′-Nucleotidase	Total	1.3	1.1	1.9	1.2	10.6	8.0	1.7	1.4
	SN	0.1	0.1	0.1	0.1	0.5	2.2	0.1	0.4
	% in SN	7.7	9.1	3.2	8.3	4.7	27.5	5.9	28.6
Na^+-, K^+-ATPase	Total	0.7	0.6	0.9	0.9	2.5	2.2	1.9	1.2
	SN	0.2	0.2	0.2	0.3	0.3	1.0	0.3	0.5
	% in SN	28.6	33.3	22.2	33.3	12.0	45.4	15.8	41.7

[a] Conditions: The cells were grown for 3 days either in the presence or in the absence of 0.5 μg/ml of prednisolone, scraped from glass with perforated cellophane, and treated with ficin as described in text. The ficin preparation contained no detectable activity of any of the enzymes assayed.

[b] Total number of units present in the preparation before incubation with ficin or buffer.

[c] Number of total units in the supernatant fluid after treatment.

[d] As related to the total activity before treatment.

activity released into the medium was approximately the same for the cells grown in the presence or in the absence of prednisolone. Again, the exception was alkaline phosphatase because, with this strain, a relatively greater amount of enzyme was released from the steroid-treated cells than from the controls. In all of the above enzyme assays, ficin, when added to the assay mixture, had no effect.

Discussion. Our data do not allow any extensive speculation about the general biochemical effect of ficin on cells in culture. The experiments were designed with hope that mild treatment of cells with proteolytic enzymes would permit some degree of separation between various membrane-associated phosphoesterase activities. Thus the observation that ficin releases a considerable amount of cell protein was expected. The results also confirmed previous observation of a protective effect of certain steroids against cellular damage (15). We reported earlier (16) that prednisolone has little selective effect on the levels of activity of phosphoesterases. The present results show that a partial separation of activities of these enzymes might be possible since the amount of activity released by ficin varies according to the enzyme. These results might also reflect the inherent properties of different cell lines. Thus in the S3G subline the alkaline phosphatase is readily released by ficin, while in the S3K line about half of the activity remains in the pellet. Prednisolone does not markedly prevent the release of this enzyme into the medium in either of the two cell lines. Leakage of other phosphoesterases, *viz.*, (Na^+, K^+)-ATPase inorganic pyrophosphatase, and 5′-nucleotidase, was observed in some instances after incubating the cells in buffer. Ficin treatment appeared only to increase leakage of these enzymes into the medium. It should be emphasized that the designation of these enzymes was based only on their activities and, since no purification was attempted, not all of the results reflect the actual quantity of the enzyme proteins released. Also a modification by ficin of the enzyme activities toward different substrates should be considered. Never-

theless, the data provide a possible new approach to the separation of various membrane-associated enzymes that should prove fruitful when large amounts of membrane material become available. Moreover, they could provide a new tool for cytochemical studies by both conventional and electron microscopy.

Summary. Two strains of HeLa cells designated as S3G and S3K were treated at 37° with a 0.4% solution of ficin. The treatment resulted in a loss of from 15 to 30% of cellular protein. Four phosphoesterases known t be associated with the plasma membrane, *viz.*, alkaline phosphatase, inorganic pyrophosphatase, (Na^+, K^+)-ATPase, and 5′-nucleotidase, were assayed after ficin treatment in the supernatant fluid and in the remaining cell pellets. In most instances, ficin substantially increased the shedding of these enzymes by the cells. Some inhibition of the protein leakage was observed in cells exposed during growth to 0.5 μg/ml of prednisolone. The loss of protein and the shedding of membrane-associated enzymes did not result in a decrease of cell viability as judged by cell cloning and dye exclusion assays.

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