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RELEASE OF MEMBRANE CONSTITUENTS FOLLOWING POLYETHYLENE GLYCOL TREATMENT OF HEp-2 CELLS

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Summary

HEp-2 cell monolayers were treated with 40% polyethylene glycol for 5 min which resulted in fusion during the subsequent incubation period. A loss of cell membrane components was detected in the polyethylene glycol-treated as well as phosphate buffer/saline-treated control cells, however the polyethylene glycol-treated cells released nearly twice the amount of [^{14}C]acetate-labeled material and [^{3}H]glycerol-labeled lipids into culture fluids than the control cells. It was further detected that the polyethylene glycol-treated cells released only approximately half the amount of protein, glycoprotein, and glycolipid as the control cells. These results suggest that polyethylene glycol exerts a differential mode of action against cell surface components and causes the treated cells to release membrane components rich in lipids but relatively low in protein and carbohydrate-containing components.

Introduction

Polyethylene glycol is increasingly being employed as a fusogen in somatic cell fusion and hybridization studies. The fusogenic action of polyethylene glycol transcends the barrier of species and types of cell membranes used. Fusion between cell membranes of plant protoplasts [1,2], animal cells [3,4], bacterial and fungal protoplasts [5,6] and even interkingdom hybrids [7,8] have been reported. Despite these demonstrations of the capability of polyethylene glycol in inducing fusion, relatively little is known about its mode of action or effect on biological membranes.

Bruckdorfer et al. [9] and Satir et al. [10], reported a blistering appearance following fusogen treatment. We have observed a similar blistered appearance following polyethylene glycol treatment of HEp-2 cells [11], however, the membrane blistering effect of polyethylene glycol was not observed by Maul et al. [12] in L cells. These conflicting observations prompted us to investigate further the effect of polyethylene glycol on cell membranes. In the present communication we report that polyethylene glycol causes a differential loss of certain membrane components into the culture medium.

Materials and Methods

Cell cultures. HEp-2 cells were grown in Eagle's minimal essential medium supplemented with 5% calf serum plus antibiotics. The cells were grown as monolayers in roller tubes or on coverslips in 35-mm petri dishes.

Polyethylene glycol treatment. Preliminary studies (unpublished) showed that polyethylene glycol (mol. wt. 1000, obtained from Olin Chemical Corp., Brandenburg, KY) at a concentration of 40% (v/v) in culture medium was optimal for fusion of HEp-2 cells. The cultures were washed with phosphate-buffered saline, pH 7.2, and overlaid with prewarmed 40% polyethylene glycol for 5 min at 37°C. The polyethylene glycol was then removed, the cultures were washed twice with phosphate-buffered saline, and fresh prewarmed culture medium was added. For cytological studies, cells on coverslips were fixed with neutral buffered formalin at various times following treatment and subsequently stained with hematoxylin and eosin. Stained cells were examined by light microscopy. Cells and nuclei from over 50 randomly chosen fields were counted. The extent of cell fusion is expressed as the fusion index:

$$\text{Fusion index} = \frac{\text{total number of nuclei—total number of cells}}{\text{total number of nuclei}} \times 100$$

Preparation of released membrane fragments. Culture media from both polyethylene glycol-treated and control cells were collected following 2 h of incubation at 37°C. The collected fluids were centrifuged at 500 × *g* for 10 min. The supernatants were carefully removed and centrifuged at 30 000 × *g* for 1 h. The pellets were collected, resuspended in 0.1 M HEPES buffer (Sigma, St. Louis, MO) pH 7.0, and homogenized with a motor-driven Teflon-coated Potter-Elvehjem homogenizer. The homogenates were centrifuged at 30 000 × *g*, for 1 h. The resulting pellets were again collected and subjected to two additional cycles of washing, homogenization and centrifugation. The final washed membranes were stored at −70°C until used.

Lipid phosphorus and protein analysis. Released membrane fragments were prepared as described. Lipids were extracted by the procedure of Bligh and Dyer [13] and chromatographed on silica gel type I, 60—200 mesh (Sigma) columns (0.5 × 3 cm). They were eluted with a chloroform/methanol (1 : 1, v/v) mixture followed by elution with methanol. The effluents were evaporated and organic phosphorus was determined by the procedure of Rouser et al. [14] as described by Fan and Jenkin [15].

The membrane protein content was determined by the procedure of Lowry et al. [16].

Isotope labeling. Washed monolayers of HEp-2 cells containing $1 \cdot 10^6$ cells/tube were labeled by incubating for 24 h in culture medium containing one of the following isotopes purchased from New England Nuclear, Boston, MA: 0.4 μCi/ml of [1-^{14}C]acetate (59 Ci/mol), 1 μCi/ml of [2-^{3}H]glycerol (6.35 Ci/mol), 1 μCi/ml D-[6-^{3}H]glucosamine (29 Ci/mol) or 0.2 μCi/ml [U-^{14}C]isoleucine (321 Ci/mol).

Prior to polyethylene glycol treatment, each culture tube was washed twice with phosphate-buffered saline. Following the polyethylene glycol treatment, cultures were washed twice with phosphate-buffered saline and incubated in fresh culture medium without labeled isotope. At intervals, cultures were centrifuged at $500 \times g$, 10 min and supernatant fluids were collected. Cells were washed once with phosphate-buffered saline and wash fluids were combined with the supernatant culture fluids. The combined fluids were again centrifuged and the radioactivity of the combined fluids quantified by liquid scintillation counting.

In the case of [^{3}H]glycerol-labeled cells the lipid was extracted from the fluids by the procedure of Bligh and Dyer [13]. Extracted lipids were dissolved in 0.05 ml methanol and mixed with 4.5 ml liquid scintillation cocktail.

For analyses of radioactivity present in macromolecules released into the culture fluid, an equal volume of 10% trichloroacetic acid solution was added to each of the combined culture fluids. The mixed suspension was left for 1 h at room temperature. The trichloroacetic acid-precipitated materials were then collected on 4.7 cm Millipore filter membranes (0.22 μm pore size, Millipore Corp., Bedford, MA). The precipitates were washed twice with 5 ml 5% trichloroacetic acid and twice with 90% methanol. Filters were dried in an oven at 50°C and counted by means of liquid scintillation.

Results

HEp-2 cells on cover slips were treated with polyethylene glycol for the determination of the extent of polyethylene glycol-induced cell fusion. Cell fusion indices reached a value of 29.5 at 1 h following treatment and remained at that approximate level over the next 24 h (Table I). The results indicated that the initial fusion event occurred within the first hour following treatment and that subsequent incubation of the treated cells did not affect the overall incidence of the fused cell population.

The effect of polyethylene glycol on the treated cells was investigated by following the release of cellular components into the medium from the treated cells during the incubation period. There are reports in the literature [17—19] that cultured cells shed membrane components following a change in medium. Results as shown in Fig. 1 and subsequent experiments demonstrated the same phenomenon in HEp-2 cells. The effect of polyethylene glycol was represented by the difference observed in the loss of radioactive components by normal cells as compared to the cells treated with polyethylene glycol. Confluent monolayers of HEp-2 cells were labeled with [^{14}C]acetate, which is essentially a mass label. An immediate release of labeled cellular components into the culture fluids from both polyethylene glycol-treated and control cells was observed after the respective changes in culture medium. The loss in the control

TABLE I

FUSION INDICES OF POLYETHYLENE GLYCOL-TREATED HEp-2 CELLS

Cells were seeded at $5 \cdot 10^4$/1.5 cm circular coverslip, incubated 24 h than treated for 5 min with 40% polyethylene glycol. Cells were then washed with phosphate-buffered saline, medium was added and cells were incubated. Fusion index was determined by the following formula: Fusion index = ((total number of nuclei — total number of cells)/total number of nuclei) X 100.

Time (h)		Nuclei/cells	Fusion index
Polyethylene glycol-treated	1	1044/736	29.5
	4	1104/762	31.0
	8	1141/800	29.9
	24	1148/823	28.3
Non-treated	4	1268/1204	5.0
	8	1038/1002	3.5
	24	1129/1093	3.2

cells began to level off about 2 h post-treatment while the treated cells continued to release materials until 4 h post-treatment. The amount of the labeled materials released by the treated cells in the first 4 h of incubation was about twice the amount observed for the control cells during the same period.

The loss of cellular lipids following polyethylene glycol treatment was determined by following [^{3}H]glycerol-labeled lipids (Fig. 2). There was a steady but increased amount of lipid released by the treated cells in the first 2 h of incubation, followed by an accelerated release which peaked at 4 h post-treatment. The control cells also exhibited a loss of lipid but the loss was much less than the polyethylene glycol-treated cells.

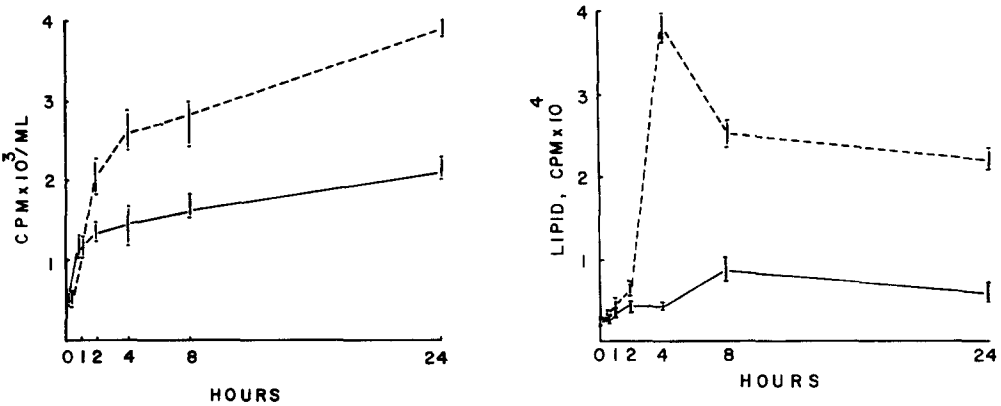

Fig. 1. The release of ^{14}C label from HEp-2 cells treated for 5 min with 40% polyethylene glycol, washed, and allowed to incubate for various time intervals post-treatment. - - - - - -, cpm from [^{14}C]acetate-labeled HEp-2 cells following polyethylene glycol treatment; ———, cpm from [^{14}C]acetate-labeled control HEp-2 cells. The range of quadruplicate samples is plotted.

Fig. 2. The release of [^{3}H]glycerol-labeled lipids from HEp-2 cells treated for 5 min with 40% polyethylene glycol, washed, and allowed to incubate for various time intervals post-treatment, - - - - - -, cpm from polyethylene glycol-treated HEp-2 cells; ———, cpm from non-treated cells. The range of quadruplicate samples is plotted.

Tritiated glucosamine was used to monitor the release of carbohydrate-containing macromolecules, mainly membrane glycoprotein and glycolipids from the polyethylene glycol-treated cells (Fig. 3). A rapid release of the labeled macromolecules was noticed immediately following the treatment in both treated and control cells. The amount of the radioactive components released by the control cells was about one-third higher than the polyethylene glycol-treated cells in the first hour following treatment. The amount of radioactive macromolecules released by both sets of cells gradually decreased after the initial hour but a moderate amount of release continued throughout the 24 h incubation period. The amount of the glucosamine-labeled macromolecules released by the polyethylene glycol-treated cells during the 24 h incubation period was about 1-fold less than that of the control cells.

The release of protein as the result of the polyethylene glycol treatment was studied by following the release of [^{14}C]isoleucine into a trichloroacetic acid-precipitable fraction present in the incubation medium (Fig. 4). A rapid release of protein was observed immediately in both the treated and control HEp-2 cells. A decline in the release of protein was observed in the polyethylene glycol-treated cells after the first hour of incubation. The amount of radioactive protein released by the treated cells gradually leveled off after 8 h of incubation while the untreated control cells continued to release protein throughout the 24 h incubation period. The amount of labeled protein released in 24 h by the control cells was about double that of the treated cells.

The protein and lipid content was determined for membranes released following polyethylene glycol treatment, and those released by control cells (Table II). For membranes released by untreated cells we found that 1.66 μg lipid phosphorus was associated with every $3.3 \cdot 10^3$ μg of membrane protein. The membranes released by HEp-2 cells following polyethylene glycol treatment contained 9.0 μg lipid phosphorus for every $4 \cdot 10^3$ μg membrane protein. Membranes released after polyethylene glycol treatment thus exhibited nearly a 4.5-fold increase in the lipid/protein ratio.

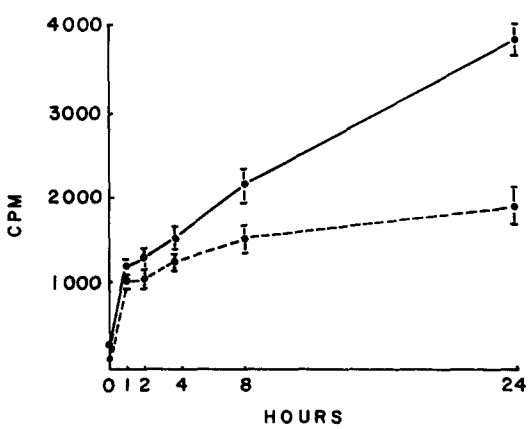

Fig. 3. The release of [^{3}H]glucosamine-labeled glycoproteins and glycolipids from HEp-2 cells treated for 5 min with 40% polyethylene glycol, washed, and allowed to incubate for various time intervals post-treatment, •------•, cpm from polyethylene glycol-treated HEp-2 cells; •———•, cpm from non-treated cells. The range of quadruplicate samples is plotted.

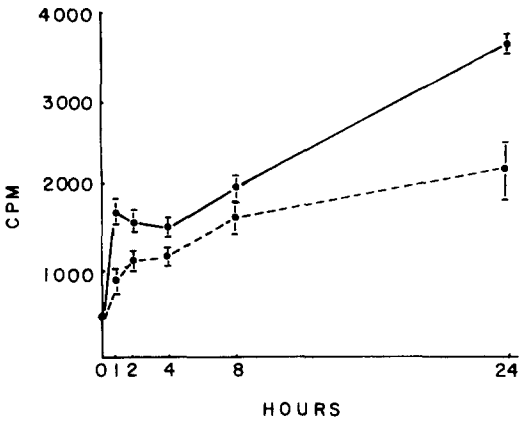

Fig. 4. Trichloroacetic acid-precipitable [^{14}C]isoleucine-labeled proteins found in the cell culture medium following treatment of HEp-2 cells, •------•, cpm from cells treated with 40% polyethylene glycol and sampled at various time intervals; •———•, cpm from control HEp-2 cells. The range of quadruplicate samples is plotted.

TABLE II

PROTEIN-LIPID RATIOS IN MEMBRANE FRAGMENTS FROM POLYETHYLENE GLYCOL-TREATED HEp-2 CELLS

	Protein (μg/ml)	Lipid phosphorous (μg/ml)	Protein/lipid *
Treated **,***	4045 ± 820	9.01 ± 0.58	20.41
Non-treated ***	3265 ± 373	1.66 ± 0.29	89.40

* An average molecular weight of 700 was used for calculating the weight of membrane lipids, μg phosphorous was multiplied by 22 to determine μg membrane lipid.

** Cells treated with 40% polyethylene glycol for 5 min.

*** Protein and lipid phosphorous values are the mean ± S.D. of data derived from six total determinations in two separate experiments.

Discussion

Cell fusion induced by polyethylene glycol is a rapid process with initial membrane fusion between adjacent cells occurring within a 5-min treatment period [12]. The fused membrane regions between cells are often difficult to detect by light microscopy or even electron microscopy [12]. Therefore it is common practice to incubate the treated cells for several hours, during which time the intracellular contents flow together and nuclei aggregate. This allows for better visual quantification of the fused cells. The post-treatment incubation does not affect the incidence of cell fusion, as shown in Table I.

Although the initial membrane fusion induced by polyethylene glycol takes place rapidly, other physiological sequelae of the treatment continue for several hours. The treated cells released large amounts of acetate-labeled material. Cell membranes of the treated cells also leak cytoplasmic components (unpublished). The release of membranes by the treated cells is evident by the

recovery of large amounts of solvent-extractable lipids from the culture fluids.

Many investigators [17—19] have shown that normal cells shed membrane components following mild changes in environment such as the addition of fresh medium. It was not surprising to detect this same phenomenon in HEp-2 cells. Of significance, however, was the differential loss of membrane components by the polyethylene glycol-treated cells. The amount of protein and carbohydrate-containing membrane components released by the polyethylene glycol-treated cells was reduced whereas the amount of lipid loss was increased compared to control cells. These results suggest that membrane fragments shed as the result of polyethylene glycol treatment have a composition quite different from those released by the untreated cells.

The lipid/protein ratio of the membranes released by these treated cells showed an increase of 4.5-fold over the membranes released by the untreated cultures. This indicates that lipid-rich membrane fragments released by polyethylene glycol-treated cells originated from membrane regions that are rich in lipids. These multiple lipid-rich regions may arise from the restricted mobilities of certain membrane proteins caused by exposure to polyethylene glycol. Aggregation of membrane protein would be involved in creating these lipid rich-protein poor regions. These altered membrane regions may then be eliminated from the cell surface and released into the culture fluid.

As a result of this study, we propose that polyethylene glycol exerts a differential effect on certain membrane components in leading to membrane fusion between cells. Though fusion proceeds rapidly during these altered membrane states [12,20,21], differential shedding of membrane components may continue for some time as a means of recovery by the treated cells.

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