Selective Cytotoxicity of Phospholipids and Diacylglycerols to Rat 3Y1 Fibroblasts Transformed by Adenovirus Type 12 or Its E1A Gene

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ABSTRACT

The colony-forming ability of rat 3Y1 fibroblasts transformed by adenovirus type 12 (Ad12) was drastically reduced when the cells were cultivated for 18 h in medium augmented with 300 μg/ml of liposomes composed of either phosphatidylcholine (PC) or phosphatidylinositol. In contrast, those of untransformed 3Y1 cells and simian virus 40-transformed and polyomavirus-transformed 3Y1 cells were not. The cytotoxicity of PC liposomes was also observed in 3Y1 cells transformed by plasmid DNA containing Ad12-E1A gene but not in those transformed by adenovirus type 2, Rous avian sarcoma virus, or plasmid DNA carrying v-Ha-ras oncogene. The extensive killing of Ad12-transformed and E1A-transformed 3Y1 cells occurred in liposomes of dioleoyl-PC and of dillinoeoyl PC but not those of dipalmitoyl PC, distearoyl-PC, or diarachidonyl PC, suggesting that the acyl groups of phospholipids play an important role in cytoxicity. Dillinoeoylglycerol, 60 μg/ml, was also cytotoxic selectively to Ad12-transformed and E1A-transformed 3Y1 cells, although the toxicity of lysophosphatidylcholine or linoleic acid was not specific to these transfectants. These results suggest that cell transformation by Ad12 is characterized by a high sensitivity to exogenously administered phospholipids and diacylglycerol that contain oleoyl or linoleoyl acyl groups and that the sensitivity is attributable to the expression of E1A gene of Ad12.

INTRODUCTION

Cancer cells and transformed cells in culture display an alteration in composition and chemical structure of membrane components such as glycoproteins, glycolipids, and phospholipids (for review, see Refs. 1–3). Rat 3Y1, an established fibroblastic cell line which behaves normally in proliferation under cell culture conditions (4), is readily transformed by various types of oncogenic viruses and oncopogenes (4–7). Ad12-transformed 3Y1 cells (Ad12-3Y1) were reported to alter their phospholipid and glycolipid composition, and the alteration seems to be relevant to malignant transformation by Ad12 (8–11). It was recently reported that Ad12-3Y1 accumulates phosphorylcholine and diacylglycerols and that the former can be easily detected by 31P nuclear magnetic resonance (11, 12). These findings suggest the occurrence of an abnormal lipid metabolism in Ad12-3Y1.

Since phospholipid vesicles (liposomes) were shown to interact with cultured cells, liposomes have been used as vehicles capable of modifying lipids of the cell membrane and of introducing various materials captured in the liposomes or dissolved in the phospholipid bilayer of liposomes into target cells (for reviews, see Refs. 13 and 14). Addition of liposomes into culture medium of various types of cells results in alteration in cell shape, cell functions, proliferation property, and survival of cells (15–21). To study the possible difference in response of the various transformed derivatives of 3Y1 cells including Ad12-3Y1 to phospholipids added exogenously, we examined the toxic effects of phospholipid liposomes on the proliferation and viability of cells. Results obtained here show that Ad12-transformed 3Y1 cells are more sensitive than other types of transformed 3Y1 cells to the cell killing mediated by phospholipids and diacylglycerols.

MATERIALS AND METHODS

Cell Cultures. Cells were cultured in plastic dishes (Nunc, Roskilde, Denmark) with Dulbecco's modified Eagle's medium (with 0.1% glucose and 0.37% NaHCO3) supplemented with 10% fetal bovine serum (Flow, Stanmore, Australia) in moist air containing 10% CO2 at 37°C (22).

Cell Lines. 3Y1-B clone 1–6 (referred to as 3Y1) is a fibroblastic cell line established from Fischer rat embryo (4), from which all the transformed rat cell lines used in this study are derived. All the transfectants show higher saturation density than parental 3Y1 cells, and all but SV-3Y1-C66 and E1A-3Y1 are capable of growth in soft agar medium. SV-3Y1-C66 and Py-3Y1-S2 are 3Y1 cells transformed by SV40 and Py, respectively (23). Ad12-3Y1 lines are 3Y1 cells transformed with Ad12 and were established by Dr. H. Zaito, Kyushu University, by infection with Ad12 virions. E1A-3Y1 lines are 3Y1 cells transformed by the EIA fragment of Ad12 genome and were isolated as follows. Proliferating 3Y1 cells were transfected with plasmid DNA containing Ad12-E1A and Ecoqst (gAE1A) (24) by the calcium phosphate method and cultivated in the medium containing 250 μg/ml of mycophenolic acid (24), and surviving colonies were isolated and cloned once. The three E1A-3Y1 lines used in this study expressed the E1A gene (see “Results”). in203S-3Y1 lines are 3Y1 cells transformed by the in203S mutant of Ad12. The in203S is a mutant of Ad12 that expressed E1A-12S mRNA but does not express normal E1A-135 mRNA because of insertion of 6 base pairs into the E1A-13S mRNA unique region (25). Resting 3Y1 cells in confluency were inoculated with in203S virions at a multiplicity of infection of 0.1–1 PFU/cell. After 2 h adsorption, the cells were replated at lower cell density (10⁴ cells/51-mm dish) and cultured for 3–4 weeks with medium changes every 7 days. Dense foci were formed against the background of the confluent untransformed cell monolayer with efficiency of (0.7–1.0) × 10⁶ PFU per focus and several were isolated. Only one focus was picked from a given culture to ensure isolation of independently transformed cell lines and cloned once. Ad2-3Y1 lines are transformed 3Y1 cell lines isolated after infection with Ad2 virions (26) at a multiplicity of infection of 5 PFU/cell. The isolation procedures were the same as for in203S-3Y1 lines described above. The efficiency of dense-focus formation was 2.3 × 10⁴ PFU/focus. HR-3Y1 lines are 3Y1 cells transformed with the v-Ha-ras oncogene and were established as follows. Proliferating 3Y1 cells were transfected with plasmid DNA containing Harvey murine sarcoma virus genome (27) by the calcium phosphate method. At 48 h after transfection, the cells were replated at low density and cultured for 3–4 weeks. Dense foci were isolated and cloned once. The HR-3Y1 lines used in this study produced a much increased amount of RNA detectable by Northern blot hybridization technique (28) using v-Ha-ras cDNA as a probe (data not shown). SR-3Y1 lines are 3Y1 cells transformed with Rous avian sarcoma virus (Schmidt-Ruppin strain subgroup D) by using 6 μg/ml of DEAE-dextran. Ad12-HTM-2 is a hamster clonal cell population obtained from the primary cultures of a tumour induced by injection of Ad12 s.c. into a newborn golden hamster. BHK21 C13 is

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The abbreviations used are: Ad12, adenovirus type 12; Ad2, adenovirus type 2; Py, mouse polyomavirus; DEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline (137 mM NaCl-2.5 mM KCl-0.8 mM NaHPO4-1.5 mM KH2PO4, pH 7.4); PFU, plaque-forming units; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.
an untransformed fibroblastic line of baby hamster kidney (29).

Chemicals. Unless otherwise specified, egg yolk phosphatidylcholine was used. All lipids listed below were purchased from Sigma Chemical Co., St. Louis, MO. The purity of the phospholipid preparations was checked by thin layer chromatography using precoated Silica Gel 60 plates (No. 5715; Merck, Darmstadt, Germany) with solvent systems of chloroform:methanol:water (65:25:4) and hexane:ether:acetate (80:30:1). When visualized by exposure to iodine vapor and/or by baking at 150°C for 60 min after spraying 50% H2SO4, 0.5% lypospholipids and 1% diacylglycerols and free fatty acids were detectable. Under these conditions, all phospholipids we used were free from contaminations of 0.5-1.0% lypospholipids, diacylglycerols, free fatty acids, or others. L-α-Phosphatidylcholine (P-5388; from egg yolk, type III-E); dipalmityl-L-α-phosphatidylcholine (P-0763); distearoyl-L-α-phosphatidylcholine (P-1138); dioleoylphosphatidylcholine (P-1013); a-dilinoleoylphosphatidylcholine (P-7649); diarachinonyl-D-α-phosphatidylcholine (P-9989); L-α-phosphatidylethanolamine (P-6386; type III, from egg yolk); L-α-phosphatidylinositol (P-0639, from soybean); L-α-phosphatidyl-ω-γ-tocopherol (P-5531, from egg yolk lecithin); sphingomyelin (S-7004, from bovine brain); L-α-lysosphatidylcholine (L-4129, type 1, from egg yolk); 1,2-dioleoyl-rac-glycerol (D-8394); 1,3-dilinoleoylglycerol (a mixture with 1,2-isomer, D-9508); 1-oleoyl-2-acetylglycerol (O-7253); linoleic anhydride (L-4628); and oleic anhydride (O-7251).

Preparation of Lipid Suspension. Phospholipids dried under a stream of N2 were swollen in Ca2+ and Mg2+-free PBS at a concentration of 1.6 mg/ml and sonicated for 60 s at 25°C unless otherwise specified (30, 31). The liposome suspension obtained was diluted with culture medium and used for treatment of cells as described below. The use of PC liposome suspension prepared in PBS supplemented with 1 mM CaCl2 and/or 1 mM MgCl2 did not influence experimental results. Diacylglycerols dried in an N2 gas flow or fatty acids were mixed with Dulbecco's modified Eagle's medium buffered with 10 mM Na2HPO4 and 1.6 mg/ml and sonicated for 60 s at 25°C.

RESULTS

Effect of Treatment of Rat 3Y1 Cells and Their Transformed Derivatives with PC Liposomes on Cell Proliferation and Survival. To examine the effect of treatment of cells with liposomes on cell proliferation in 3Y1 and their transformed sublines, the proliferating cultures of 3Y1, SV-3Y1, Ad12-3Y1, and E1A-3Y1 were added in the medium with varying doses of PC liposomes, and the number of cells attached on the culture dish was determined at intervals (Fig. 1). Ad12-3Y1 and E1A-3Y1 degenerated and were detached from the dishes within 1 day after addition of PC liposomes of 300–400 μg/ml, whereas proliferation of 3Y1 was inhibited only slightly at 400 μg/ml and that of SV-3Y1 was not affected. Then, we examined survival of cells after treatment with PC liposomes by determining the cellular ability of colony formation (Fig. 2). All of the independently established three clones of Ad12-3Y1 and three clones of E1A-3Y1 lost colony-forming ability after incubation with 200 μg/ml PC liposomes for 18 h. Time course experiments of the selective cell killing by PC liposomes revealed that the colony-forming ability of E1A-3Y1 decreased to 1/25 at 6 h and 1/250 at 12 h after addition of 200 μg/ml of PC liposomes. We examined the cytotoxicity of PC liposomes in a wider range of transformed derivatives of 3Y1 and an Ad12-induced hamster tumor cell line (Table 1). In the presence of 300 μg/ml of PC liposomes, all clones of Ad12-3Y1, E1A-3Y1, and in203-3Y1 (3 clones for each) lost colony-forming ability, whereas all clones of SV-3Y1, Py-3Y1, Ad2-3Y1 (3
Table 1  Selective cytotoxicity of phosphatidylcholine liposomes to cells transformed by Ad12 and by Ad12 E1A gene

Cells in proliferating cultures were incubated with 300 μg/ml phosphatidylcholine liposomes for 18 h, and viable cell number was determined by colony formation assay. Survival was expressed as percentage of control in the absence of phospholipid.

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<th>Cell line</th>
<th>Transforming agent</th>
<th>Survival (% of colony formers)</th>
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<tr>
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<tr>
<td>Rat</td>
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| Hamster     |                  |               |               |               |               |               |
| BHK21 C13   |                   |               |               |               |               | 90             |
| Ad12-HTM-2  | Ad12            | 0.01          |               |               |               |               |

* not determined.

in203S, a mutant of Ad12.

RSV, Rous avian sarcoma virus, strain Schmidt-Ruppin D.

Possible differences in the amount of cellular uptake of the liposomal phospholipids, PC liposomes (200 μg/ml) were prepared with a trace amount of radioactive [14C-choline]PC and incubated with 3Y1 and E1A-3Y1 for 2 h at 37°C. Cell-associated lipids were almost equal in both cell lines, approximately 120 ng/10^6 cells (3Y1, 0.24 μg/2.0 x 10^6 cells; E1A-3Y1, 0.30 μg/2.6 x 10^6 cells), suggesting that the selective toxicity of PC to E1A-3Y1 is not due to difference in amount of lipids associated with cells.

The effect of different acyl groups of PC on the cytotoxicity was examined. Egg yolk PC is a mixture of PC composed of various acyl groups. Reportedly, the fatty acid compositions are palmitic acid (32%), stearic acid (16%), oleic acid (30%), linoleic acid (17%), and arachidonic acid (3%) (36). We prepared liposome suspensions composed of synthetic PC contain-
ing different acyl groups, and the cytotoxicity of these liposomes was examined (Table 3). Dioleoyl-PC and dilinoleoyl-PC were more toxic to Ad12-3Y1 and E1A-3Y1 than to 3Y1 but neither distearoyl-PC, dipalmitoyl-PC, nor diarachidonyl-PC were toxic at this same dose. Remarkably, liposomes of dilinoleoyl-PC were so cytotoxic that a dose as low as 90–120 µg/ml was sufficient to impair the viability of Ad12-3Y1 and E1A-3Y1.

Cytotoxic Effect of Various Lipids on Ad12-3Y1 and E1A-3Y1. To know whether Ad12-3Y1 is sensitive to lipids other than phospholipids, we examined the cytotoxicity of diacylglycerols, lysophospholipids, and free fatty acids. Diacylglycerols, which are supposed to be produced by digestion of phospholipids by phospholipase C in the cells, were added to the culture medium, and cell survival was determined (Fig. 4). Dilinoleoylglycerol was more toxic to Ad12-3Y1 and E1A-3Y1 at 40–120 µg/ml than to 3Y1, whereas dioleoylglycerol did not show cytotoxicity in 3Y1, Ad12-3Y1, and E1A-3Y1 even at 300 µg/ml. To 1-oleoyl-2-acetylglycerol, Ad12-3Y1 was more sensitive than 3Y1, and E1A-3Y1 was far more sensitive. As expected, dilinoleoylglycerol, similar to PC liposomes, was not toxic for SV-3Y1, Py-3Y1, HR-3Y1, and SR-3Y1 at 120 µg/ml (data not shown). Lysophospholipids and free fatty acids are supposed to be produced by digestion of phospholipids by phospholipase A2. When lysophosphatidylcholine was added to the culture, viability of Ad12-3Y1, E1A-3Y1, and 3Y1 was equally impaired at 8–10 µg/ml (Fig. 5). In the case of fatty acids, linoleic acid was equally cytotoxic in Ad12-3Y1, E1A-3Y1, and 3Y1 at 100–120 µg/ml, whereas oleic acid was not toxic even at 160 µg/ml for all the cell lines. These results indicate, although lysophospholipids and linoleic acid were toxic to Ad12-transformed 3Y1 cells as reported in other types of cells (37, 38), that the higher sensitivity of Ad12-3Y1 and E1A-3Y1 to lipids is confined to phospholipids and diacylglycerols.

**DISCUSSION**

We found in this study that Ad12-transformed cells were more sensitive to cell killing by phospholipid liposomes and diacylglycerols than the untransformed counterpart. The high sensitivity of cells to PC liposomes seems to be not a common character of transformed cells but a specific event in Ad12-transformed cells, because 3Y1 cells transformed by such a variety of viruses and oncogene other than Ad12 as SV40, Py, Ad2, Rous sarcoma virus, and v-Ha-ras gene had as low sensitivity as untransformed 3Y1 cells did.

Since Ad12-3Y1 was highly sensitive to PI liposomes, as well as to PC liposomes, the difference in the polar head groups of phospholipids is not critical for the cytotoxicity. Rather, the acyl groups of lipids play an essential role, because liposomes of dilinoleoyl-PC or dioleoyl-PC were more toxic to Ad12-3Y1 and E1A-3Y1 than to 3Y1, but those of dipalmitoyl-PC, distearoyl-PC, and diarachidonyl-PC were not (Table 3). Jett and Alving (21) reported that plant PI liposomes are selectively cytotoxic to several human cancer cell lines whereas animal PI liposomes are not. Since plant PI contains more unsaturated acyl chains than animal PI, especially linoleic acid in the sn-2 position (39), liposomes composed of phospholipids containing certain sorts of unsaturated acyl chains may have selective killing activity not only in Ad12-transformed cells as reported here but also in some types of human cancer cells.

The mechanism of killing of Ad12-transformed cells by phospholipid liposomes is unclear. One possible explanation is that exogenously administered phospholipids would be incorporated into cells and metabolized, and the metabolized product(s) would be toxic for Ad12-transformed cells. It is known that phospholipid liposomes became associated with cell surface and exogenously administered phospholipids would be incorporated into cells and metabolized, and the metabolized product(s) would be toxic for Ad12-transformed cells. It is known that phospholipid liposomes became associated with cell surface and exogenously administered phospholipids would be incorporated into cells and metabolized, and the metabolized product(s) would be toxic for Ad12-transformed cells.
fusion between liposomal membrane and cellular membrane, or by a combination of these events (40–44). In the case of PC liposomes in fluid state, PC is incorporated into cells by endocytosis and by lipid transfer (40, 42). Our present results that PC added to the cell cultures became associated with cells with a similar efficiency in Ad12-transformed 3Y1 cells and untransformed 3Y1 cells and that diacylglycerols were also toxic as well as phospholipids, suggest that the differential cell killing observed in Ad12-transformed cells may not be due to differences in cellular uptake of phospholipids. Jett et al. (45) demonstrated that lysophospholipids, free fatty acids, and diacylglycerols are released in significant quantities from PI liposomes after incubation with neuroblastoma cells. They claimed that the products may be responsible for selective cell killing by PI liposomes (45). Ad12-transformed 3Y1 cells were reported to accumulate diacylglycerols and phosphorylcholine (11, 12). Our present finding that diacylglycerols but neither lysophatidylcholine nor linoleic acid were also toxic selectively to Ad12-transformed cells suggests that the selective cytotoxicity may be due to diacylglycerols per se. In this regard, it is of interest that diacylglycerol modifies protein kinase C activity and may promote cell proliferation (46, 47). However, there is another possibility that linoleic acids would be released abundantly in Ad12-transformed cells by phospholipase A2 or diacylglycerolase.

The higher sensitivity of Ad12-3Y1 to phospholipids and diacylglycerols probably results from Ad12-E1A gene expression. E1A gene of Ad12 was reported to be required for cell transformation and induction of cellular DNA synthesis in cells quiescent at confluent cell density (6, 24). It was reported that E1A gene products act in trans as an enhancer of the transcription of certain viral and cellular genes and act as a suppressor of the transcription of the cellular gene of a histocompatibility antigen (48–50). It may be possible that the altered lipid metabolism in Ad12-3Y1 might be a result of alteration in regulation or function of some cellular proteins participating in lipid metabolism, which may be caused by the Ad12-E1A gene products.

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