Pharmaceutical Biology
1998, Vol. 36, No. 2, pp. 111–123

1388-0209/98/3602-0111$12.00

SOY (*GLYCINE MAX*)-DERIVED PHOSPHOLIPIDS EXHIBIT POTENT ANTI-APOPTOTIC ACTIVITY

I.C. Bathurst*, J.D. Bradley**, J.G. Goddard, M.W. Foehr, J.P. Shapiro, P.J. Barr*** and L.D. Tomei

LXR Biotechnology Inc., 1401 Marina Way South, Richmond, CA 94804, USA

ABSTRACT

*We have isolated and identified a soybean phospholipid mixture from unroasted soy (*Glycine max*) flour that is a potent inhibitor of apoptotic cell death. This phospholipid mixture has been purified from several soy derived materials including soy flour, soy molasses, and crude commercial soy lecithin. Analysis of this bioactive lipid mixture has identified the two major constituents as phosphatidic acid and phosphatidylinositol. This lipid mixture also contains lesser amounts of lysophosphatidic acid, lysophosphatidylinositol, and lysophosphatidylcholine. These phospholipids contain the typical distribution of fatty acids found in soy, predominantly C16:0 and C18:2 (hexadecanoic and 9,12-octadecadienoic) in a 60:40 to 50:50 ratio. Less than 10% of other varieties of fatty acids were identified; the most common other fatty acids found were C18:0, C18:1 and C18:3. Apoptosis inhibition was assessed following serum deprivation of a mouse embryonic stem cell line (C3H-10T1/2). This anti-apoptotic bioassay was used to monitor the purification of the bioactive phospholipid mixture. Of the phospholipids contained in the mixture, lysophosphatidic acid was found to be the most potent inhibitor of apoptotic cell death. The anti-apoptotic factor described here is distinct from the bioactive, well characterized estrogen-like, anti-cancer factor and the Bowman-Birk protease inhibitor, neither of which possess anti-apoptotic activity in this assay.*

Keywords: Apoptosis, C3H-10T1/2 cells, lysophosphatidic acid, phospholipids, serum deprivation, soy.

Latin binomials: *Glycine max.*

* Author to whom correspondence should be addressed.
** Present address: Scriptgen Pharmaceuticals, Inc., 610 Lincoln Street, Waltham, MA 02154, USA.
*** Alphaone Pharmaceuticals, Inc., 850 Marina Village Parkway, Alameda, CA 94501, USA.

INTRODUCTION

The term “apoptosis” was proposed more than 20 years ago to describe an active process of controlled cell deletion (Kerr et al., 1972). Apoptosis, also called programmed cell death, has been described in many biological systems and has been shown to be important in ontogeny as well as in the maintenance of healthy tissues in adult animals (Kerr et al., 1980). Apoptotic death is commonly marked by cellular shrinkage, chromatin condensation, increased membrane permeability, and inter-nucleosomal DNA cleavage (Kerr et al., 1972, 1980; Tomei, 1991; Umansky, 1982; Wyllie, 1980). As a consequence of recent interest in the phenomenon of apoptosis, a substantial number of publications have appeared in which current understanding of the phenomenology and physiology of programmed cell death is reviewed (Umansky, 1996; Vaux & Strasser, 1996; Steller, 1995; Thompson, 1995). Recent studies of apoptosis have implied that a common final biochemical pathway leading to cell death may be initiated by a wide variety of signals including those associated with hormones, serum and/or growth factor deprivation, chemotherapeutic agents, ionizing radiation and infection by human imunodeficiency virus (HIV) (Tomei et al., 1993; Hickman, 1992; Kanter & Schwartz, 1980; Carson & Rebeiro, 1993). Perhaps the most important feature to emerge from recent apoptosis research is the fact that novel therapeutic development strategies are possible that can lead to drug intervention when pathogenesis of diseases are marked by acute onset of apoptosis in specific tissues and organs (Tomei et al., 1993).

Several reports in the literature indicate that some seeds may contain a factor or factors that are capable of inhibiting apoptosis. The pre-feeding of soy flour or soy flour extracts to rats has been shown to alleviate the diarrhea, weight loss, and the decreased food intake

associated with methotrexate (MTX) administration (Funk & Baker, 1991a,b). Similarly, specific diets containing soy fractions have been shown to alleviate intestinal damage caused by gamma irradiation in a dog model (McArdle et al., 1985, 1986). In each of these examples, the cytotoxic effect on intestinal epithelial cells has been attributed to induction of apoptotic pathways (Pinkerton et al., 1982; Hickman, 1992). Additionally, certain soy-based food formulations are capable of lessening the diarrhea and wasting associated with some stages of AIDS (Chlebowski et al., 1993, 1995).

Recently the bioactive lipid lysophosphatidic acid (LPA) has been shown to be the major non-protein species in serum which modulates proliferation, differentiation, and cytoskeletal architecture in a number of different cell culture systems (Moolenaar, 1995). At the molecular level, LPA has been shown to operate through a pertussis toxin sensitive G_i protein which mediates the mitogenic effects through activation of the Ras-MAP kinase pathway (Howe & Marshall, 1993). LPA also stimulates a pertussis toxin-insensitive G_q protein(s) which activates the phosphatidylinositol-phospholipase C pathway resulting in release of intracellular calcium stores and activation of diacylglycerol responsive PKC (van Corven et al., 1989). In addition, LPA effects a Rho-dependent formation of actin stress fibers, tyrosine phosphorylation of the $p125^{FAK}$ focal adhesion kinase (and some related proteins) and increased focal adhesion production (Ridley & Hall, 1992; Seufferlein & Rozengurt, 1994). Recently, our laboratory reported the cloning of a functional LPA membrane receptor (Gou et al., 1996).

Like LPA, lysophosphatidylcholine (LPC) has also been shown to possess biological activities, partially blocking cytotoxic effects of some ether lipids (Boggs et al., 1995) and facilitating normal fibroblast cell cycle progression (Terce et al., 1994). More recently, a genetic defect in phosphatidylcholine (PC) biosynthesis has been shown to trigger apoptosis in CHO cells (Cui et al., 1996).

In this study we present results related to the development of a potential therapeutic apoptosis modulator. The experimental results demonstrate that a soy (*Glycine max*)-derived phospholipid mixture, free from protein, phosphatidylcholine (PC), and phosphatidylethanolamine (PE), but containing phosphatidic acid (PA), phosphatidylinositol (PI), lysophosphatidic acid (LPA), lysophosphatidylinositol (LPI), and lysophosphatidylcholine (LPC) is capable of successfully inhibiting apoptosis.

MATERIALS AND METHODS

Extraction and Isolation

Soy (*Glycine max*) flour, type 1 (Sigma, St. Louis, MO) was defatted by stirring at room temperature for 30 min in 70% acetone (250 g/l). The defatted soy flour was recovered by centrifugation at 2,000 x g for 10 min at 4°C, and the anti-apoptotic factor extracted into 50% ethanol (125 g/l) by stirring at room temperature for 1 h. Following centrifugation at 3,000 × g for 10 min at 4°C to remove the solids, the supernatant was diluted with an equal volume of water. The ethanol was removed and activity concentrated by ultrafiltration over a 10 kilodalton membrane (Pall-Filtron Technologies Inc., Boston, MA). This material was lyophilized, tested for activity, and further purified by resuspending in water: methanol: chloroform (3:8:4, vol./vol./vol.) as described by Bligh and Dyer (1959). The insoluble material was removed by centrifugation (10,000 × g for 20 min at 4°C) and the soluble fraction containing the anti-apoptotic activity was reclaimed by rotary evaporation and stored dry or dissolved in chloroform: methanol (4:1) at −20°C. This water: methanol: chloroform (3:8:4) soluble material is referred to as the soy lipid fraction of the acetone/ethanol extract.

Further purification of the active soy lipid fraction was achieved by normal phase silicic acid chromatography using silicic acid 100 mesh (Mallinckrodt Chemical Inc., Paris, KT) under gravity at room temperature equilibrated with chloroform: methanol (4:1). Material dissolved in chloroform: methanol (4:1) was loaded onto the pre-equilbrated column and washed with 5 volumes of the equilibration solvent. The active fractions were eluted either with a gradient to chloroform: methanol (1:4) or by sequential steps of 4:1, 3:2, 2:3, and finally 1:4, chloroform: methanol. The active fractions were chosen according to the anti-apoptosis bioassay (described below) and pooled for further characterization and purification. Normal phase HPLC using a Dionex system (Sunnyvale, CA) consisting of a GP40 pump, AD20 detector and an 8 μm silica Dynamax® 250 × 10 mm column (Rainin Instrument Co., Woburn, MA) at a flow rate of 4 ml/min was used to further purify the active fractions. Two different gradient solvent systems were then utilized; the first from chloroform:methanol (4:1) to chloroform: methanol: 30% ammonium hydroxide (6:4:1) over 40 min at 4 ml/min, and the second from chloroform:methanol (4:1) to chloroform: acetone: methanol: acetic acid: water (6:8:2:2:1), also over 40 min at 4 ml/min. Both gradients were held at 100% of the second solvent for

10 min before reconditioning the column with the initial solvent conditions. All fractions collected were 8 ml. Because the measured absorbance at 260 nm was complicated by interference by the chloroform in the mobile phase, the primary means of monitoring the elution of the lipids was thin layer chromatography (TLC).

Thin Layer Chromatography

One- and two-dimensional TLC were used to analyze the lipid composition during the purification process. K6, 60Å, 250 µm silica plates (20 × 20 cm) obtained from Whatman (Clifton, NJ) were stored desiccated at room temperature. First dimensions were performed in chloroform: methanol: ammonium hydroxide (6:4:1) and after air drying, the second dimensions were separated using chloroform: acetone: methanol: acetic acid: water (6:8:2:2:1). After final drying, the plates were developed either for total lipids by spraying with 50% sulfuric acid followed by heating for 20 min at 110°C or for phospholipids by spraying with the Zinzadze's reagent using molybdic oxide and molybdenum in a sulfuric acid, acetic acid, water mixture (Henderson & Tocher, 1992). Identification of individual lipids was confirmed initially by comigration with commercial phospholipid standards and their relative quantities were estimated by scanning/densitometry.

Anti-Apoptotic Bioassay

The C3H-10T1/2 cell apoptotic assay was performed as described by Tomei et al. (1993). Briefly, mouse fibroblast C3H-10T1/2 cells (clone 8) were obtained from ATCC (Rockville, MD) and maintained in exponential growth phase where the cell cycle is randomly distributed and were not permitted to reach density-dependent cell cycle arrest. Due to increased risk of spontaneous loss of the untransformed phenotype, all assays were performed on cells only up to serial passage 15.

Cells from cultures maintained in log phase were seeded in 60 mm petri dishes at 175 to 350 cells per cm^2 in Basal Media Eagle (BME) (Gibco BRL, Grand Island, NY) with Earle's salts and L-glutamine supplemented with 10% fetal calf serum (Hyclone, Logan, UT). Five days and 2 days prior to initiating the assay a fresh media change occurred. At $T = 0$ the cultures were typically 60–80% confluent and their media was replaced with serum-free BME media to induce apoptosis. Test samples were resuspended in serum free BME media and sterile filtered. Controls included the addition of 5×10^{-8} M 12-o-tetradecanoyl phorbol-13-acetate (TPA) (Sigma, St. Louis, MO) to ensure the responsiveness of the cell culture to an anti-apoptotic signal. All assays were performed in triplicate and analyses were made 18 to 24 h after serum deprivation. Two parameters were analyzed on each cell culture plate. All non-adherent or loosely adherent cells were removed from the culture dish and measured by counting in a electric particle counting instrument (Coulter Corporation, Hialeah, FL) with a lower threshold of 6.9 µm. These serum deprived released cells (SDR) were defined as the apoptotic fraction released in response to cultivation in serum-free medium. Approximately 95% of these released cells are apoptotic as shown by both size, ultrastructure and DNA fragmentation analysis (Tomei et al., 1993). The remaining adherent cells (ADH) were treated with Hanks balanced salt solution without calcium and magnesium salts containing trypsin (0.05%) and ethylenediamine tetraacetic acid, EDTA (0.53 mM). Each culture was incubated at room temperature on a rocking platform to ensure uniform distribution of the trypsin reagent over the surface. After 10 min, the cells were removed from each culture dish and measured as described for the non-adherent cells with a lower threshold set at 11 µm.

Cell Staining and Photography

Cells were visualized directly in the tissue culture dish, under phase contrast and after staining for fluorescence using a mixture of propidium iodide (Sigma, St. Louis, MO) at 1 µg/ml of culture media and bisbenzimide Hoechst 33342 (Sigma, St. Louis, MO) at 10 µg/ml of culture media. Photography was performed at least 15 min after the addition of the staining solution. Color 35 mm slides of the tissue culture cells were made at 20 × magnification with phase contrast under UV and visible illumination using a Nikon Diaphot-200 fluorescence microscope equipped with a high pressure mercury lamp, UV filter block and an FX-II 35 mm camera (Nikon, Garden City, NY).

Fatty Acid Analysis

Determination of the type and ratios of the fatty acids present were performed as the fatty acid methyl esters using the trans-esterification reagent, anhydrous methanolic HCl (Aldrich, Milwaukee, WI) as described by Christie (1987, 1989). To each sample, 300 µl of dichloromethane and 700 µl of hydrochloric acid/methanol was added. Derivatization was performed under nitrogen at room temperature for 18 h. After incubation, 1 ml of water was added and the samples were extracted three times with 2 ml of hexane. The combined extracts were dried under a stream of nitrogen, redissolved in 100 µl of hexane and transferred to gc-ms

vials. Analysis of samples was performed on a Hewlett-Packard 5890 gas chromatograph with a Hewlett-Packard 5971 series mass selective detector (Hewlett-Packard, Avondale, PA) as described by Van Den Berg et al. (1993).

Mass Spectroscopy

Compounds of interest were identified and extracted from preparative silica TLC plates (Whatman, Clifton, NJ) using chloroform:methanol (1:1). Electrospray mass spectroscopy was performed on a VG BloQ Triple quadrupole mass spectrometer with electrospray ionization in the negative mode. The source temperature was 80°C and the solvent was methanol or methanol with 0.05% ammonium acetate at a flow rate of 5 µl/min. The capillary voltage was 4.7 kV and analysis performed as described by Christie (1987).

Preparation of Commercial Lipid Mixture (Reconstituted Natural Mixture)

Pure (99%) soy phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and were combined in a ratio similar to that seen in the purified bioactive fraction in order to attempt to reconstitute the naturally occurring soy material. The reconstituted lipid mixture was dried from a chloroform solution and resuspended in the anti-apoptosis bioassay media by 5 min of sonication in a Branson 200 bath sonicator (Branson Ultrasonics Corporation, Danbury, CT).

Mitogenic Activity

The mitogenic activity of the crude, purified or reconstituted phospholipids was assayed in a similar manner to that described by Tigyi and Meledi (1992). The C3H-10T1/2 cell assay was performed using a [6-^{3}H] Thymidine (925 GBq/mmol) (Amersham Life Science, Arlington Heights, IL) pulse during the final 6 h prior to counting the cells. Cells (both the SDR and ADH) were harvested onto a 2.4 cm glass fiber disc (GF/C Whatman, Clifton, NJ), washed with phosphate buffered saline and once with ethanol before being air dried. Counts associated with the various cell fractions were analyzed using Ready-Safe scintillation fluid in a LS5801 liquid scintillation counter (Beckman Instruments, Irvine, CA).

RESULTS

In an *in vitro* assay system of cultured C3H-10T1/2 cells undergoing logarithmic growth, removal of serum typically leads to initiation of apoptosis (programmed cell death) in 60–70% of the cells within 24 h. Using this assay we have identified an anti-apoptotic activity present in soy flour.

As described in Table 1, this anti-apoptotic activity can be purified from unroasted soy flour after a 70% acetone extraction is used to remove soluble apoptotic activity (see lines 1 and 2, Table 1). Extraction of the acetone insoluble material with 50% ethanol solublized the anti-apoptotic activity and during removal of the ethanol, the aqueous extract first turned opalescent before finally becoming cloudy. Upon drying, approximately 50% of the ethanol extracted material and all of the anti-apoptotic activity was soluble in chloroform: methanol: water (4:8:3) suggesting a phospholipid or glycolipid nature to the active substance(s) (Bligh & Dyer, 1959).

Large scale, low pressure silicic acid chromatography equilibrated in chloroform:methanol (4:1) was initially used to remove phosphatidylcholine (PC), phosphatidylethanolamine (PE) and some free fatty acids from the active fraction, which was then eluted with chloroform: methanol (1:4). Both the soy lipid fraction of the acetone/ethanol extract or the active fraction from the silicic acid chromatography could be further purified by semi-preparative normal phase silica HPLC using a gradient from chloroform: methanol (4:1) to chloroform: methanol: 30% NH_4OH (6:4:1) as described in *Materials and Methods*. This procedure resolved PC and PE from a group of phospholipids that eluted between 32 and 44 min and contained all of the anti-apoptotic activity. These fractions were pooled, dried, and rechromatographed over the same column eluting with a gradient from chloroform: methanol (4:1) to chloroform: acetone: methanol: acetic acid: water (6:8:2:2:1). An elution profile of this chromatographic step is shown in Figure 1 both as sulfuric acid charred (lower panel) and Zinzadze stained (upper panel) one-dimensional thin layer chromatograms. The phospholipids identified by their relative R_f values were PA, LPA, PI, LPI, and LPC.

Also shown in Figure 1 is the anti-apoptotic activity of each fraction. In general, all of the anti-apoptotic activity of the starting material could not be recovered from the sum of the individual phospholipids unless fatty acid free bovine serum albumin (BSA) (Boehringer Mannheim) was used to assist in resuspending and presenting the phospholipid (Tigyi & Meledi, 1992).

Following the identification of five of the phospholipids involved in the activity, commercially pure soy

Table 1. Characterization of soy (*Glycine max*) anti-apoptotic activity at different stages of purification.[a]

	Yield	Anti-apoptotic Activity	Anti-trypsin Activity	A^{260}	A^{280}	% Nucleic acid
Soy Flour Water Extract	196 mg/gm	Apoptotic	0.561 U/ug	116/gm	134/gm	2.4%
Soy Flour 70% Acetone Extract	20 mg/gm	Apoptotic	ND	4.5/gm	7.2/gm	11%
Soy Flour Acetone Pellet 50% Ethanol Extract	10 mg/gm	106 ug/ml	177 U/ug	32/gm	36/gm	ND
Soy Flour Acetone Pellet 50% Ethanol Extract Organic Extract ("Soy Lipid Fraction")	5 mg/gm	63 ug/ml	ND	<1/gm	<1/gm	ND
Soy Flour Acetone Pellet 50% Ethanol Extract Organic Extract Silicic acid Eluate	1.6 mg/gm	21 ug/ml	ND	ND	ND	ND
Soy Flour Acetone Pellet 50% Ethanol Extract Organic Extract Silicic acid Eluate hplc Pool	300 ug/gm	6 ug/ml	ND	ND	ND	ND

ND = Not detected

[a] Anti-apoptotic activity is measured as the dose of material necessary to inhibit 50% of the cell death that would normally occur following 24 hours of serum deprivation. Trypsin inhibitory activity was measured using an enzyme substrate assay as described by Abramovitz et al. (1983). Nucleic acid concentration was estimated comparing relative spectrophotometric absorbance readings.

lipids were purchased and combined in a ratio similar to that observed in bioactive samples (PA:PI:LPA:LPI:LPC–10:10:2:2:1). Bioactivities of each of the extracts and the reconstituted mixture are compared to LPA + 0.01% BSA in Figure 2.

Cell staining and photography confirmed the inhibition of apoptosis and showed a decrease in the number of cells permeable to propidium iodide (i.e. apoptotic cells). Cultures were treated with either the lipid fraction of the acetone/ethanol extract or with the mixture of commercially available pure soy phospholipids combined in a similar ratio as that seen in the lipid extract (PA:PI:LPA:LPI:LPC–10:10:2:2:1) (Fig. 3).

Fractions eluted from the HPLC (Figure 1) or extracted from duplicated unstained TLC plates with methanol were subjected to electrospray mass spectroscopy as described in *Materials and Methods*. Electrospray mass spectroscopy of extracts (Figs. 4 A–E) confirmed the lipid identities to be PA (4A), PI (4B), LPA (4C), LPI (4D), and LPC (4E). For all five lipids, primary mass peaks correlated with predetermined known weights and overall mass spectra matched those seen with pure soy lipid controls. The profiles for four of the five lipids purified exhibited a peak 24 mass units from the major peak, indicating the presence of two distinct species likely differing in chain length and degree of saturation such as the difference between a palmitoyl and linoleoyl fatty acid residue.

Fatty acid analysis (Table 2) of samples purified from the lipid fraction as described in the *Materials and Methods* section indicated a mixture of C16:0 and C18:2 (hexadecanoic and 9,12-octadecadienoic) and C18:1 and C18:0. Phosphatidylinositol (PI) was identified by atomic weight, nmr, and co-migration with authentic standards on TLC. The fatty acid composition was similar to that obtained for LPA of C16:0 and C18:2 (hexadecanoic and 9,12-octadecadienoic). These fatty acid ratios are common for soy derived phospholipids (Avanti Polar Lipids Product Catalog, 1994).

When assayed in the presence of 0.01% BSA, LPA

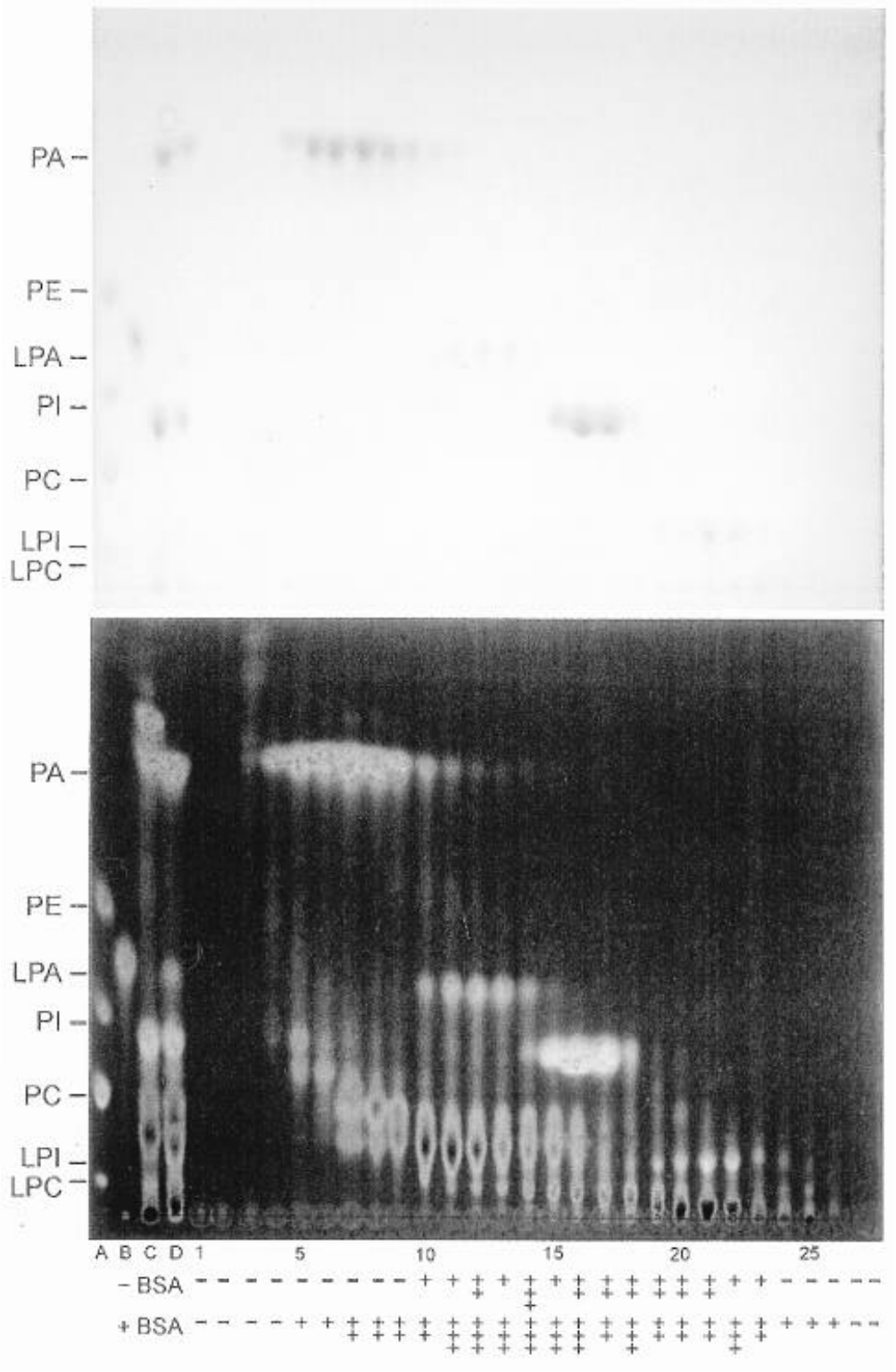

Fig. 1. Thin layer chromatography profiles of soy (*Glycine max*) extracts and anti-apoptotic activity derived from silica HPLC using a gradient from chloroform: methanol (4:1) to chloroform: acetone: methanol: acetic acid: water (6:8:2:2:1); visualized using Zinzadze stain for phospholipid phosphate (upper panel) and 50% sulfuric acid charring (lower panel). Anti-apoptotic activity of each fraction is depicted using $+++ => 40\%$ cells remaining viable; $++ = 30$–40% cells remaining viable; $+ = 20$–30% cells remaining viable; $- =< 20\%$ cells remaining viable. Lane A is a soy phospholipid standard mixture containing LPC, PC, PI and PE. Lane B contains LPA, Lane C is the organic soluble 50% ethanol extract and Lane D is the pool of active fractions from the first silica hplc separation as detailed in *Materials and Methods*. Lanes 1 to 25 are samples from the 8 ml fractions collected.

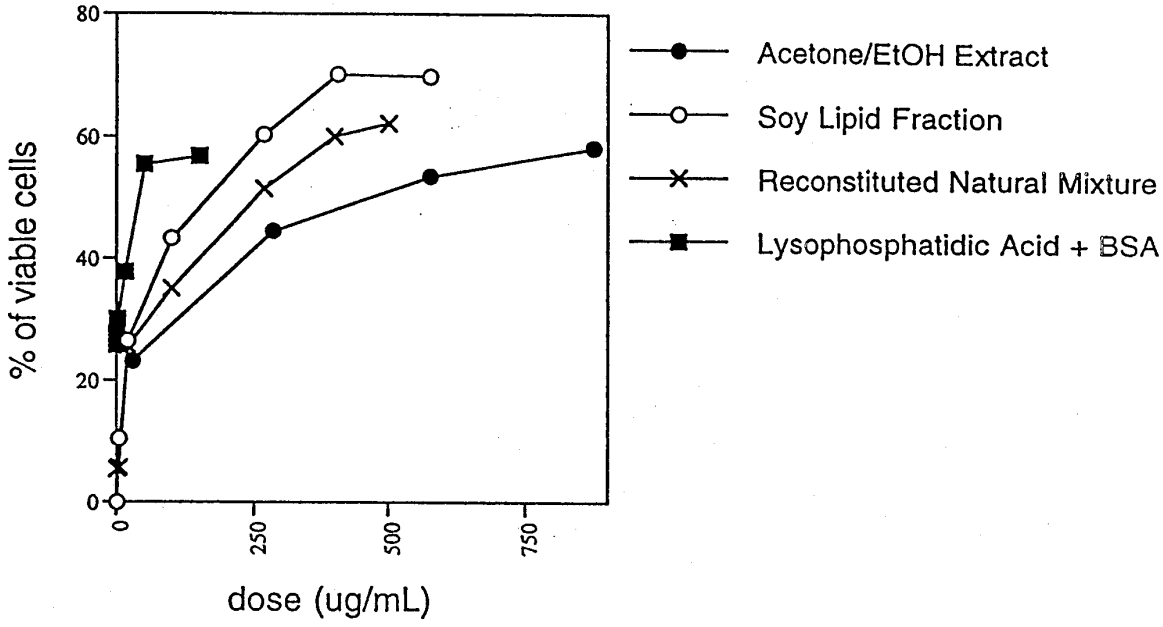

Fig. 2. Anti-apoptotic activity of soy (*Glycine max*) extracts at various doses. Two of the active fractions described in Table 1 were tested for their maximal activity of saving C3H-10T1/2 cells from serum deprivation induced apoptosis. Commercially available phospholipids combined in a similar ratio to that found in the silica purified fractions was compared to LPA administered with 0.01% fatty acid free bovine serum albumin.

possessed the most potent anti-apoptotic activity, with the ability to save over half of the cells that would otherwise die by an apoptotic mechanism (Fig. 2, Table 3). LPC also contained anti-apoptotic activity, saving 28%. The other phospholipid components in the lipid extract (PA, PI, LPI) as well as PE, PG, and LPE posses a small amount of anti-apoptotic activity when administered with 0.01% BSA (Table 3). Both PC and LPG increased cell death in the C3H-10T1/2 cells.

It has been shown previously in the literature that LPA possesses a mitogenic effect on some cell lines (van Corven et al., 1989, 1992; Piazza et al., 1995). Tests for mitogenic activity showed that neither the acetone/ethanol extract nor the lipid fraction of the acetone/ethanol extract caused an increase in thymidine uptake over control cultures (Fig. 5). The reconstituted natural phospholipid mixture exhibited more mitogenicity than the extracts but significantly less than LPA alone or a TPA control.

Figure 6 illustrates the time course of apoptosis exhibited by the C3H-10T1/2 cells and the apoptotic inhibition by the lipid fraction when cells are counted at various times following treatment. Cells in this assay began dying by apoptosis as early as 6 h continuing through 18 h following serum deprivation. In the presence of the soy lipid fraction, cell death is reduced from

Table 2. Summary of fatty acid distribution data from gc-ms analysis of silica HPLC purified lipids.[a]

Lipid(s) Purified	Fatty Acid Composition (mol %)			
	Palmitoyl (16:0)	Linoleoyl (18:2)	Oloeyl (18:1)	Steroyl (18:0)
PA	63	31	2	4
PA	46	47	4	3
PA, PI, LPA	43	46	6	5
LPA	32	45	10	14
LPC, LPI	41	46	5	7
PI, LPA*	76	0	0	24

[a] Fraction noted with "*" was purified from a soy lecithin. All others are HPLC purified fractions from soy flour.

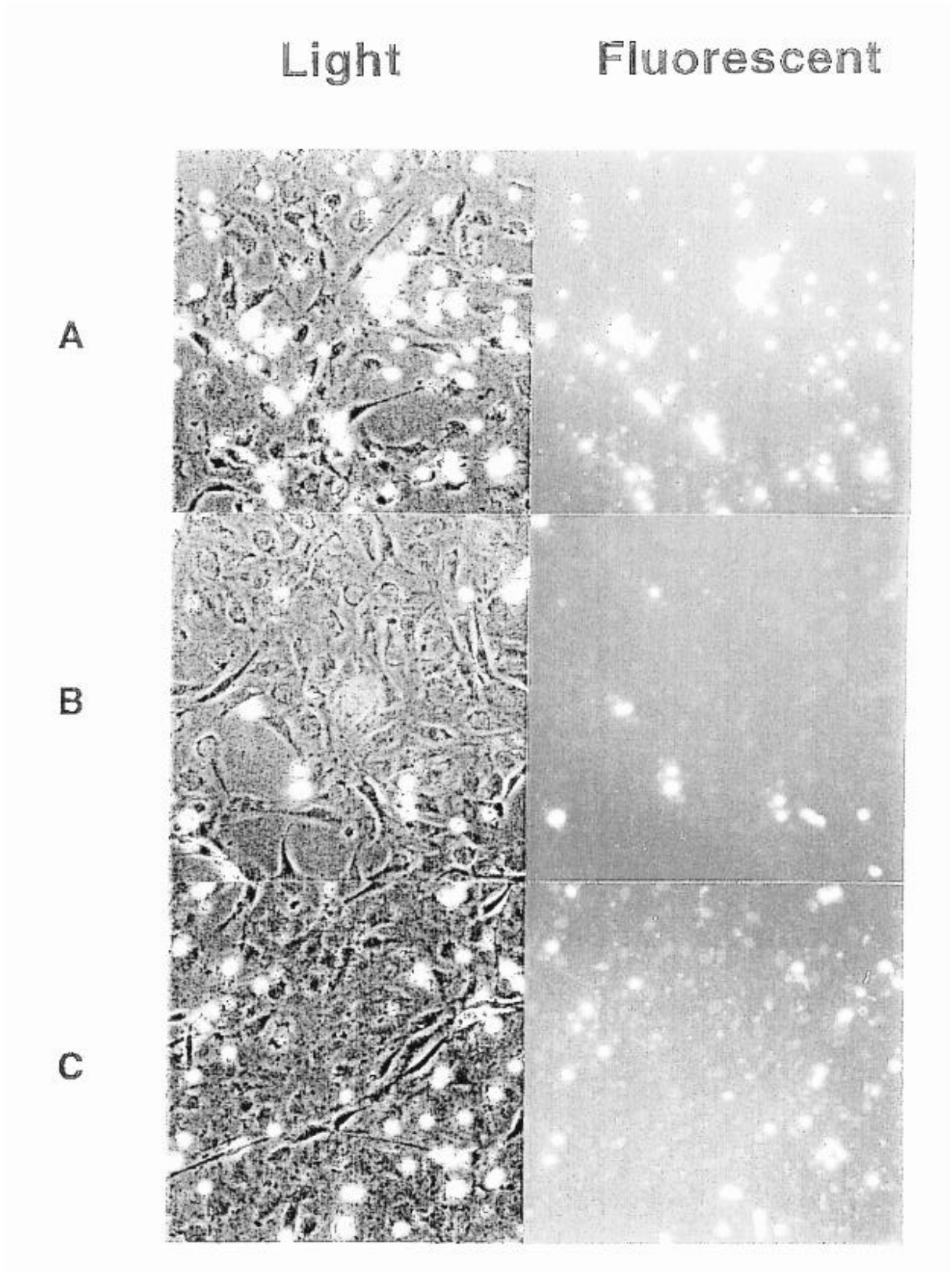

Fig. 3. Light and florescence microscopy photographs demonstrating the bioactivity of the natural and commercial reconstituted soy (*Glycine max*) – derived anti-apoptotic activity; (A) serum-free control; (B) soy lipid fraction of acetone/ethanol extract; (C) reconstituted natural phospholipid mixture.

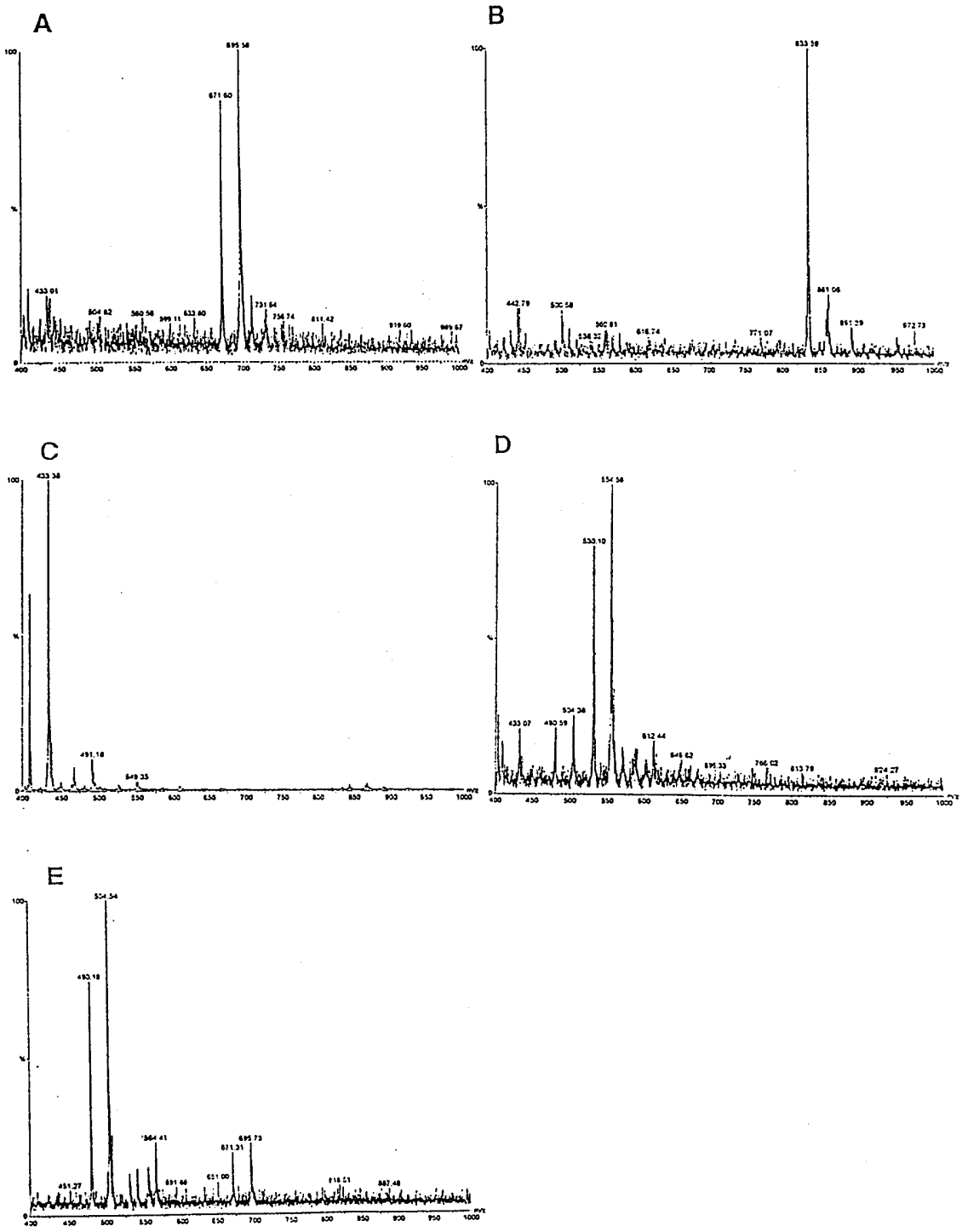

Fig. 4. (A–E) Electrospray mass spectroscopy profiles of phospholipids extracted from TLC silica. Molecular weights correlate with pure lipid tlc migration for PA (A); PI (B); LPA (C); LPI (D); LPC (E).

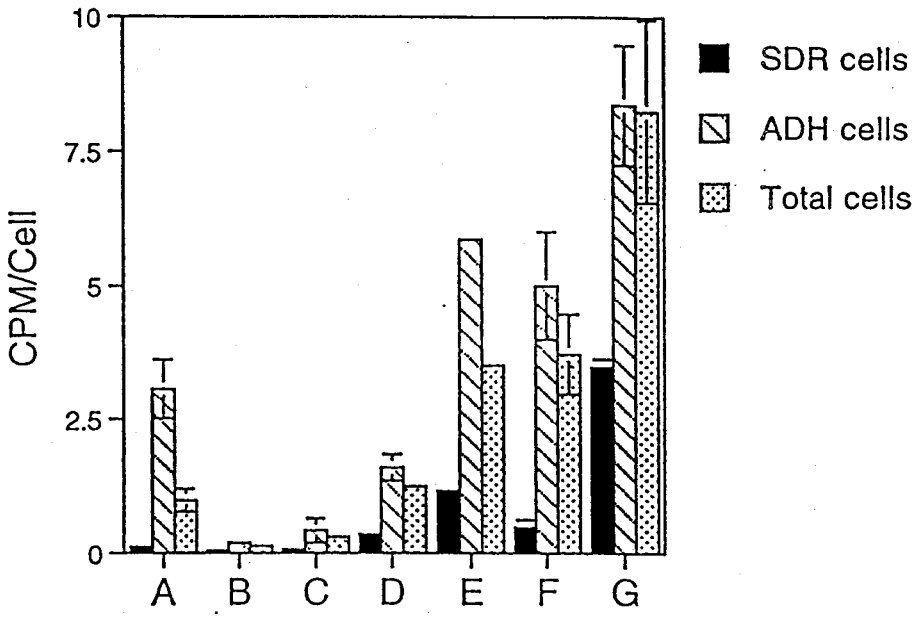

Fig. 5. Measurement of the incorporation of [6-^{3}H] Thymidine by C3H-10T1/2 cells exposed to the soy (*Glycine max*) – derived anti-apoptotic mixtures as compared to controls. Cells were pulsed for 8 hours (from 16 hours post treatment to 24 hours post treatment). Compared, are (A) serum free control; (B) the acetone/ethanol soy flour extract; (C) soy lipid fraction of acetone/ethanol extract; (D) reconstituted natural phospholipid mixture; (E) soy derived lysophosphatidic acid with 0.01% fatty acid free BSA; (F) 12-O-tetradecanoyl phorbol-13–acetate/TPA; and (G) a serum control.

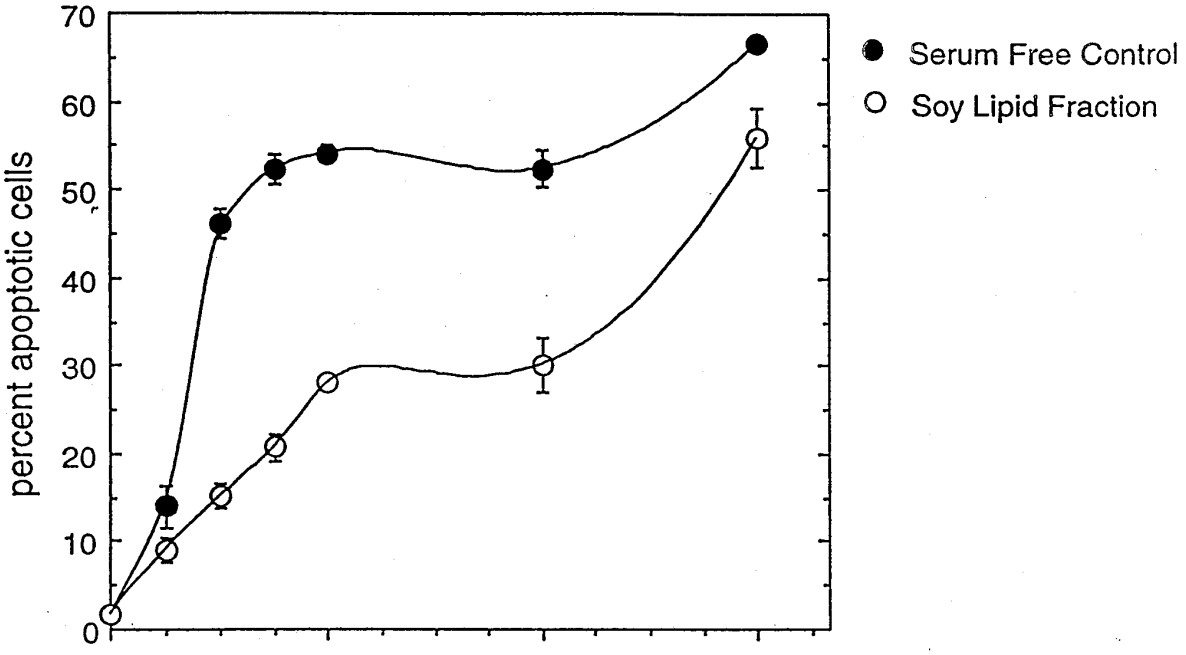

Fig. 6. Protection from apoptosis at various times following the addition of the soy (*Glycine max*) - derived anti-apoptotic lipid fraction of the acetone/ethanol extract to serum deprived C3H-10T1/2 cells. Serum withdrawal and addition of the anti-apoptotic factor occurred at time zero.

Table 3. Activity of various pure phospholipids as measured by the prevention of cell death in the C3H-10T1/2 cell assay.[a]

Phospholipid	Percent of cells remaining viable
PA	2.4 ± 0.41
PC	–0.8 ± 0.94
PE	5.1 ± 0.49
PG	2.5 ± 0.42
PI	1.8 ± 0.93
LPA	58.7 ± 1.12
LPC	27.8 ± 0.05
LPE	7.6 ± 1.33
LPG	–30.3 ± 1.78
LPI	4.7 ± 0.66

[a] All phospholipids were tested at a final dose of 25 μg/ml with 0.01% fatty acid free BSA. Components present in the active soy lipid extracts described are seen in bold. Samples in which treatment resulted in increased cell death are shown with a negative value.

6 h through to 48 h, after which the protection provided by the soy lipid fraction decreased and the cell death increased.

DISCUSSION

We have screened seed flours for both pro-apoptotic and anti-apoptotic factors using an *in vitro* assay system which permits selective determination of apoptosis modulator activity in cultured untransformed C3H-10T1/2 cells. This cell type has been found to exhibit *in vitro* regulation of proliferation, pluripotent differentiation, as well as apoptosis and has been used as a model system for studies of normal physiological cell cycle control and malignant transformation (Tomei, 1991; Tomei et al., 1993). Using this *in vitro* model system, we have successfully isolated, identified, and purified a potent anti-apoptotic activity from soy flour.

The purification scheme involved delipidation of the ground soy with 70% acetone followed by an extraction into 50% ethanol. This material is heat stable (100°C for 30 min) and resistant to proteases (trypsin, chymotrypsin, proteinase K, and subtilisin), nucleases (RNAase A and DNAase), and in aqueous solutions shows the characteristics of a liposome. Purification to individual phospholipids was achieved by extraction into a single phase chloroform:methanol:water mixture followed by high performance chromatography over silica using gradient elution from chloroform:methanol first to chloroform:methanol containing ammonium hydroxide then to and acidic organic mixture. Purity was monitored by one- and two-dimensional tlc using sulfuric acid charring and/or a phospholipid specific spray for visualization.

This purified bioactive soy lipid fraction contains less than 0.05% protein, and does not have any detectable protease inhibition activity. A semi-purified soy derived Bowman-Birk protease inhibitor (BBI) has known anti-cancer activity and is currently being tested in a clinical trial as a possible oral anti-cancer therapeutic. Pure BBI exhibits no anti-apoptotic activity in our assay. Further studies are in progress to characterize the phospholipid-dependent apoptosis modulator pathways and to determine the mechanism of the anti-apoptotic activity.

The semi-purified material containing a mixture of phospholipids showed potent anti-apoptotic activity even when administered at up to 6 h following serum withdrawal (data not shown). This suggests that anti-apoptotic intervention may be initiated at some point after the apoptotic insult has occurred. The mechanism of action of the phospholipid mixture has not yet been elucidated however we have recently cloned a functional LPA membrane receptor from *Xenopus* oocytes (Gou et al., 1996). In addition to this receptor, other LPA G protein linked receptors have also been identified (An et al., 1997; Yatomi et al., 1997). Further analysis of the signal transduction pathways regulated by these and other receptors will lead to a greater understanding of possible control points within apoptosis and anti-apoptosis pathways.

We are currently evaluating an array of possible clinical applications for this mixture including reduction of apoptotic cell death associated with acute global and regional ischemia and reperfusion, organ transplantation, control of undesirable effects associated with existing anti-cancer therapies, reversal of gastrointestinal dysfunction in individuals infected with human immunodeficiency virus (HIV) and for the treatment of other diseases where acute apoptosis dysfunction may be a contributing factor.

NOTE IN PROOF

While this manuscript was in review, Levine et al. published data showing that lysophosphatidic acid acts as a growth or “survival” factor for primary cultures of mouse renal proximal tubular cells deprived of serum. (Levine YS, Koh JS, Triaca V, Lieberthal W (1997): Lysophosphatidic acid: a novel growth and survival factor for renal proximal tubular cells. *Am J Physiol* 273: 575–585).

REFERENCES

Abramovitz AS, Yavelow J, Randolf V, Troll W (1983): Inhibition of superoxide production in human neutrophils by purified soybean polypeptides: re-evaluation of the involvement of proteases. *J Biol Chem 258:* 15153–15157.

An S, Dickens MA, Bleu T, Hallmark OG, Goetzl E (1997): Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem Biophys Res Comm 231:* 619–622.

Avanti Polar Lipids, Inc (1994): *Product Catalog.* Avanti Polar Lipids, Inc., Alabaster AL.

Bligh EG, Dyer WJ (1959): A rapid method of total lipid extraction and purification. *Can J Biochem Physiol 37:* 911–914.

Boggs KP, Rock CO, Jackowski S (1995): Lysophosphatidylcholine attenuates the cytotoxic effects of the antineoplastic phospholipid 1-o-octadecyl-2-o-methyl-rac -glycero-3-phosphocholine. *J Biol Chem 270:* 11612–11618.

Carson DA, Ribeiro JM (1993): Apoptosis and disease. *Lancet 341:* 1251–1254.

Chlebowski RT, Beall G, Grosvenor M, Lillington L, Weintraub N, Ambler C, Richards EW, Abbruzzese BC, McCamish MA, Cope FO (1993): Long-term effects of early nutitional support with new enterotropic peptide-based formula vs. standard enteral formula in HIV-infected patients: randomized prospective trial. *Nutrition 9:* 507–512.

Chlebowski RT, Grosvenor M, Lillington L, Sayre J, Beall G (1995): Dietary intake and counseling, weight maintenance, and the course of HIV infection. *J Am Diet Assoc 95:* 428–432.

Christie WW (1987): The analysis of fatty acids. In: *HPLC and Lipids.* New York, Pergamon Press Inc., pp. 133–168.

Christie WW (1989): Gas chromatographic analysis of fatty acid derivatives. In: *Gas Chromatography and Lipids: A Practical Guide.* Ayr, Scotland, Oily Press Ltd., pp. 86–128,

Cui Z, Houweling M, Chen MH, Record M, Chap H, Vance DE, Terce F (1996): A genetic defect in phosphatidylcholine biosynthesis triggers apoptosis in Chinese hamster ovary cells. *J Biol Chem 271:* 14668–14671.

Funk MA, Baker DH (1991a): Effect of fiber, protein source and time of feeding on methotrexate toxicity in rats. *J Nutr 121:* 1673–1683.

Funk MA, Baker DH (1991b): Effect of soy products on methotrexate toxicity in rats. *J Nutr 121:* 1684–1692.

Gou Z, Liliom K, Fischer DJ, Bathurst IC, Tomei LD, Kiefer MC, Tigyi G (1996): Molecular cloning of a high-affinity receptor for the growth factor-like lipid mediator lysophosphatidic acid from *Xenopus* oocytes. *Proc Natl Acad Sci USA 93:* 14367–14372.

Henderson RJ, Tocher DR (1992): Thin layer chromatography. In: Hamilton RJ, Hamilton S, eds., *Lipid Analysis: A Practical Approach*. New York, Oxford University Press, pp. 66–111.

Hickman JH (1992): Apoptosis induced by anticancer drugs. *Cancer Metas Rev 11:* 121–139.

Howe LR, Marshall CJ (1993): Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring $p21^{ras}$ and $p74^{raf-1}$. *J Biol Chem 268:* 20717–20720.

Kanter P M, Schwartz HS (1980): Post-repair DNA damage in x-irradiated cultured human tumor cells. *Int J Radiat Biol Relat Stud Phys Chem Med 38:* 483–493.

Kerr JFR, Wyllie AH, Currie AR (1972): Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer 26:* 239–257.

Kerr JFR, Wyllie AH, Currie AR (1980): Cell death: the significance of apoptosis. *Int Rev Cytol 68:* 251–306.

McArdle AH, Reid EC, Laplante MP, Freeman CR (1986): Prophylaxis against radiation injury: the use of elemental diet prior to and during radiotherapy for invasive bladder cancer and in early postoperative feeding following radical cystectomy and ileal conduit. *Arch Surg 121:* 879–885.

McArdle AH, Wittnich C, Freeman CR, Duguid WP (1985): Elemental diet as prophylaxis against radiation injury: histological and ultrastructural studies. *Arch Surg 120:* 1026–1032.

Moolenaar WH (1995): Lysophosphatidic acid, a multifunctional phospholipid messenger. *J Biol Chem 270:* 12949–12952.

Piazza GA, Ritter JL, Baracka CA (1995): Lysophosphatidic acid induction of transforming growth factors alpha and beta: modulation of poliferation and differentiation in cultured human keratinocytes and mouse skin. *Exp Cell Res 216:* 51–64.

Pinkerton CR, Cameron CHS, Sloan JM, Glasgow JFT, Gevarea NJT (1982): Jejunal crypt cell abnormalities associated with methotrexate treatment in children with acute lymphoblastic leukaemia. *J Clin Path (London) 35:* 1272–1277.

Ridley AJ, Hall A (1992): The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell 70:* 389–399.

Seufferlein T, Rozengurt E (1994): Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130. *J Biol Chem 269:* 9345–9351.

Steller H (1995): Mechanisms and genes of cellular suicide. *Nature 267:* 1445–1449.

Terce F, Brun H, Vance DE (1994): Requirement of phosphatidylcholine for normal progression through the cell cycle in C3H-10T1/2 fibroblasts. *J Lipid Res 35:* 2130–2142.

Thompson CB (1995): Apoptosis in the pathogenisis and treatment of disease. *Nature 267:* 1456–1462.

Tigyi G, Meledi R (1992): Lysophosphatidates bound to serum albumin activate membrane currents in *Xenopus* oocytes and neurite retraction in PC12 pheochromocytoma cells. *J Biol Chem 267:* 21360–21367.

Tomei LD (1991): Apoptosis: a program for death or survival? In: Tomei LD, Cope FO, eds., *Apoptosis: The Molecular Basis of Cell Death*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, pp. 279–316.

Tomei LD, Shapiro JP, Cope FO (1993): Apoptosis in C3H-10T1/2 mouse embryonic cells: evidence for internucleosomal DNA modification in the absence of double-strand cleavage. *Proc Natl Acad Sci (USA) 90:* 853–857.

Umansky SR (1982): The genetic program of cell death: hypothesis and some applications, transformation, carcinogenesis, and ageing. *J Theor Biol 97:* 591–602.

Umansky SR (1996): Apoptosis: molecular and cellular mechanisms (a review). *Mol Biol 30:* 285–295.

Van Corven EJ, Groenink A, Jalink K, Eichholtz T, Moolenaar WH (1989): Lysophosphatidate-induced cell polifferation: identification and dissection of signaling pathways mediated by G proteins. *Cell 59:* 45–54.

Van Corven EJ, van Rijswijk A, Jalink K, van der Bend RL, van Blitterswijk WJ, Moolenaar W H (1992): Mitogenic action of lysophosphatidic acid and phosphatidic acid on fibroblasts: dependance on acyl-chain length and inhibition by suramin. *Biochem J 281:* 163–169.

Van den Berg JJM, Winterbourn CC, Kuypers FA (1993): Hypochlorous acid-mediated modification of cholesterol and phospholipid: analysis of reaction products by gas chromatography-mass spectromtry. *J Lipid Res 34:* 2005–2012.

Vaux DL, Strasser A (1996): The molecular biology of apoptosis. *Proc Natl Acad Sci (USA)* 93: 2239–2244.

Wyllie AH (1980): Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature 284:* 555–556.

Yatomi Y, Yamamura S, Ruan F, Igarashi Y (1997): Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J Biol Chem 272:* 5291–5297.

Accepted: January 2, 1998