[CANCER RESEARCH 48, 1066–1071, March 1, 1988]

A Putative New Growth Factor in Ascitic Fluid from Ovarian Cancer Patients: Identification, Characterization, and Mechanism of Action[1]

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ABSTRACT

Ascitic fluid from ovarian cancer patients (n = 16), but not from patients with other cancers or with benign diseases, contains a growth-promoting activity which induces the proliferation of both fresh ovarian cancer cells (n = 5) and the ovarian cancer cell line HEY. The ascitic fluid growth factor(s) appears to signal cells through binding and activation of specific, saturable, high-affinity cell surface receptors. Incubation of fresh or cultured ovarian cancer cells with a partially purified preparation of ascitic fluid stimulates phosphatidylinositol turnover and increases cytosolic-free calcium. Each of these biochemical events has been implicated in the action of growth factors. Purified preparations of previously identified growth factors including epidermal growth factor, transforming growth factor-β, tumor necrosis factor, platelet-derived growth factor, thrombin, insulin, interleukin-1, interleukin-2, vasopressin, angiotensin, α- and γ-interferons, and fibroblast growth factor did not increase cytosolic-free calcium in either fresh ovarian cancer cells or HEY cells. Therefore, ascitic fluid appears to contain one or more previously unidentified growth factors which activate ovarian cancer cells through phosphatidylinositol hydrolysis and resultant changes in cytosolic-free calcium.

INTRODUCTION

The mechanisms leading to the proliferation of malignant cells are poorly understood. Some tumors may arise by activation of oncogenes which are cellular genes involved in cell transformation by retroviruses and in spontaneous tumors (reviewed in Refs. 1–3). Other tumors may be initiated by inappropriate production or action of growth factors which are polypeptide hormones which induce the proliferation of normal cells (2, 3). Most oncogene products are components of growth factor activation pathways which suggests that these two mechanisms for the initiation of tumors may overlap (1–3).

The cause of proliferation of human ovarian cancer tumor cells, the primary cause of death from gynecologic tumors, has not been determined. Abnormal oncogene expression or action has been detected in a minority of ovarian tumors (4–7). Therefore, abnormal action of a growth factor might be involved in the growth of ovarian cancer. To test this possibility, we determined whether ascitic fluid from ovarian cancer patients contains a growth factor which induces the proliferation of ovarian cancer cells.

Ascitic fluid from ovarian cancer patients, but not from patients with benign disease or other cancers, induces proliferation of fresh ovarian cancer cells and the ovarian cancer cell line HEY (8). This proliferative response is associated with rapid increases in phospholipid hydrolysis and changes in $[Ca^{2+}]_i$. Previously characterized growth factors did not increase $[Ca^{2+}]_i$ in either fresh ovarian cancer cells or HEY cells. Ascitic fluid also increased $[Ca^{2+}]_i$ in rat mesangial cells, human breast cancer cells, and murine fibroblasts but not in lymphoid cells. Taken together, the data suggest that ascitic fluid from ovarian cancer patients contains one or more previously uncharacterized growth factors which stimulate cells through phospholipid hydrolysis.

MATERIALS AND METHODS

Media and Stock Solutions. Purified EGF was obtained from Sigma Chemical Co. (St. Louis, MO) and from Collaborative Research (Lexington, MA). PDGF, recombinant TGF-β, and FGF (acidic) were obtained from R and D Systems Inc. (Minneapolis, MN). Purified PDGF was also obtained from Sigma and Calbiochem (San Diego, CA). Purified recombinant interleukin-2 was a kind gift of Biogen Research Corp. (Cambridge, MA). Purified recombinant α- and β-interferons were from Schering (Kenilworth, NJ). Purified recombinant interleukin-1 was from Hoffmann LaRoche (Nutley, NJ). Purified recombinant TNF-α was from Genentech, Inc. (South San Francisco, CA). Insulin was from Connaught Laboratories (Toronto, Canada) and Sigma. Thrombin was from Sigma and Boehringer Mannheim (Dorval, Canada). Complete medium was RPMI 1640 (GIBCO, Grand Island, NY) substituted with 5% (v/v) FCS (Flow Laboratories, Maclean, VA), 2 mM glutamine (GIBCO), and 5×10^5 M mercaptoethanol (Sigma). Buffer A was in mM: NaCl, 140; KCl, 1; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; and HEPES, 20, pH 7.23 (Sigma). [^{3}H]Thymidine (6.7 Ci/mmol) was from New England Nuclear (Lachine, Quebec). Indo-1 was from Molecular Probes (Eugene, OR). Ionomycin was from Calbiochem. Unless stated otherwise, all other reagents were from Sigma.

Patient Selection. Ascitic fluid was obtained from patients (n = 16) undergoing laparotomy for initial diagnosis or from patients having ascitic fluid removed for palliation following completion of therapy. All patients had either mucinous or serous cystadenocarcinomas of epithelial origin.

Cells. HEY ovarian carcinoma cells (Dr. R. Buick, Toronto, Canada; Ref. 8) were carried in complete medium. These cells are epithelial in morphology, grow as a monolayer on plastic, and have a high cloning efficiency. These cells formed tumors in nude mice. Cells were split weekly. HEY cells were cultivated in RPMI 1640 without fetal calf serum for at least 48 h prior to use. Cells were harvested in trypsin 0.1% containing Ca^{2+} and Mg^{2+}-free phosphate-buffered saline supplemented with 2 mM EDTA. Ascitic fluid cells were harvested by centrifugation at 1000 rpm followed by separation on Ficoll-hypaque (Pharmacia, Uppsala, Sweden).

[^{3}H]Thymidine Incorporation. HEY cells were starved by a 48-h incubation in serum-free complete medium. Cells were harvested and incubated at 1×10^4 cells/ml in 200 μl of medium in 96-well flat-bottomed plates (Costar) with the indicated supplements for an additional 48 h. [^{3}H]Thymidine incorporation was determined by a 6-h pulse with 1 μCi/well followed by harvesting and scintillation counting.

Colony Formation. 1×10^3 HEY or $3–5 \times 10^4$ fresh ovarian cancer cells were incubated in a single-layer methylcellulose system (1% in Iscove's modified Dulbecco's medium without FCS). Iscove's modified

Received 8/11/87; revised 11/20/87; accepted 12/1/87.

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[1] Supported by grants to G. B. M. from the National Cancer Institute of Canada, the Medical Research Council of Canada, and the Genesis Foundation of Canada.

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[3] The abbreviations used are: $[Ca^{2+}]_i$, cytosolic-free calcium concentrations; EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; IP_3, inositol-1,4,5-trisphosphate; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor.

Dulbecco's medium was more efficient than RPMI 1640 in supporting growth of ovarian cancer cells. Each plate was screened 48 h later and plates exhibiting cell clumping were eliminated from the analysis. Colonies were counted 14–21 days after seeding. Colonies of greater than 15 cells were counted as positive.

$[Ca^{2+}]_i$ Measurements. For $[Ca^{2+}]_i$ measurements, cells (5×10^6/ml) were incubated with the acetoxymethylester derivative of Indo-1 (2.5 μg/ml; Refs. 9 and 10) for 20 min at 37°C in RPMI 1640 in 5% CO_2. Cells were washed 2 times and resuspended in RPMI 1640 at 1×10^6/ml. Cells were washed in Buffer A immediately prior to use. The fluorescence emission was measured in a stirred, heated cuvette in a Hitachi F 4000 spectrophotometer with excitation at 331 nm and emission at 410 nm. Following equilibration to a stable baseline, ascitic fluid or growth factors were added as described. For determination of Ca^{2+} dependence studies, cells were suspended in nominally Ca^{2+}-free Buffer A and EGTA (0.5 mM added as indicated). Each run was calibrated by addition of 5 μM ionomycin followed by 1 mM Mn^{2+}. Ca^{2+} concentration was determined as described (9, 10). The K_m for Indo-1 at pH 7.2 is 180.

Inositol Phosphate Production. HEY cells or fresh ovarian cancer cells were incubated overnight in inositol-free RPMI (Media Preparation Services, Ontario Cancer Institute, Toronto, Canada) supplemented with glutamine, mercaptoethanol, and 10% FCS. Cells (10^6) were incubated with 100 μCi of [^{3}H]inositol (Amersham) for 4 h in inositol-free RPMI (GIBCO). [^{3}H]Inositol-loaded cells were then incubated with 1% (v/v) of partially purified ascitic fluid for the indicated times. Cells were lysed with chloroform methanol (1:1) and inositol phosphates (aqueous phase) isolated by high-performance liquid chromatography on a Mono Q anion exchanger (Pharmacia) or passage through open ion exchange columns (Dowex 1X8; see Ref. 11 for details).

RESULTS

Growth-promoting Activity in Human Ovarian Cancer Ascitic Fluid. Ascitic fluid from ovarian cancer patients induced proliferation of the ovarian cancer cell line HEY as indicated by[^{3}H]-thymidine incorporation (Table 1). Maximal proliferation of HEY cells occurred at about 1% (v/v) ascitic fluid with significant proliferation detected at 0.1% ascitic fluid (Table 1). Higher concentrations of ascitic fluid were slightly inhibitory either because of dilution of nutrients in the RPMI 1640 or the presence of inhibitory factors. Higher concentrations of FCS gave somewhat higher levels of [^{3}H]thymidine incorporation than did ascitic fluid (Table 1). In contrast, the growth-promoting activity of FCS diluted out more rapidly than did ascitic fluid (Table 1). Addition of bovine serum albumin did not alter the proliferative response in the presence or absence of ascitic fluid or FCS, indicating that protein was not a limiting factor.

Incubation of HEY cells with ascitic fluid (1%) from 9 ovarian cancer patients (Table 2) resulted in maximal proliferation, indicating that ascitic fluid is a potent source of growth-promoting activity. All ascitic fluids tested gave similar levels of proliferation with similar concentration dependence (not presented). In contrast, ascitic fluid from a patient with a malignant breast tumor or a patient with benign hepatic disease did not induce proliferation of HEY cells (Table 2) even at 10-fold higher concentrations (not presented). Ascitic fluid from a patient with a malignant lung tumor induced a lower but significant level of proliferation of HEY cells (Table 2). FCS (1%), a standard source of growth factor activity, and human plasma (1%) both supported proliferation of HEY cells.

As indicated by colony formation, ascitic fluid induced proliferation of fresh ovarian cancer cells and HEY cells (Table 3). Colony formation in methylcellulose is considered to be an indicator of the proportion of the ovarian cancer stem cells which proliferate *in vivo* (12, 13). Ascitic fluid from 3 different patients had similar activities on 3 different ovarian cancer cell samples (Table 3). Therefore, ascitic fluid from ovarian cancer patients provides a potent source of growth-promoting activity which is sufficient to stimulate the proliferation of both fresh and cultured ovarian cancer cell lines. Since the growth-promoting activity was effective on HEY cells and on ovarian cancer cells from different patients, the growth factor was not restricted to autologous tumor in its activity. It is remarkable that ascitic fluid induced significant proliferation and colony formation, even at ascitic fluid concentrations 100- to 1000-fold (1 to 0.1% v/v) lower than the concentration to which cancer cells are exposed in the patient.

Ascitic fluid did not induce colony formation or [^{3}H]thymidine incorporation in lymphoid cells isolated from peripheral blood (10, 11) or from ascitic fluid (not presented). This suggests that the growth factor activity of ascitic fluid will not induce proliferation of all cell types.

Purification and Characterization. The crude ascitic fluid was acid treated and clarified by centrifugation and the majority of human serum albumin and protease activity removed on an agarose blue column. The unbound fraction from blue agarose was absorbed onto a DEAE-Sephacel blue column and eluted with 500 mM NaCl. The eluate was ammonium sulfate precipitated (50–90% saturation), reconstituted in buffer, and dialyzed against Buffer A. This partial purification results in a 1000-fold increase in specific activity with little loss of total activity. This partially purified ascitic fluid was utilized for all subsequent experiments. As indicated in Table 2, the partially purified ascitic fluid was as effective as fresh ascitic fluid or FCS in inducing proliferation of ovarian cancer cells. The ascitic fluid growth factor activity was acid (pH 2 for 30 min) and trypsin (1%; 37°C for 3 h) stable. Incubation with protease K (37°C for 3 h, 200 μg/ml) removed activity. Activity was relatively heat stable. No activity was lost at 56°C for 30 min; 50% of the activity was lost at 70°C for 30 min and 100% of the activity

Table 1 *Comparison of growth-promoting activity of ascitic fluid and FCS*

HEY cells were starved of FCS for 48 h, harvested, and plated at 1×10^4 cells/ml in 96-well microtiter plates. Cells were incubated for 48 h in the presence and absence of the indicated concentrations of ascitic fluid from one patient or FCS with and without 1 mg/ml bovine serum albumin as a protein source. Cells were labeled with [^{3}H]thymidine for 6 h, harvested, and counted by liquid scintillation. Data represent means ± SEM for 6 replicates; one of 5 experiments with ascitic fluid from different patients is shown.

Concentration added (v/v) (%)	[^{3}H]Thymidine incorporation (cpm) of ascitic fluid		[^{3}H]Thymidine incorporation (cpm) of FCS	
	−BSA	+BSA	−BSA	+BSA
10	13,190 ± 1,640	15,530 ± 2,080	22,530 ± 1,660	20,060 ± 1,620
1	17,780 ± 1,410	14,680 ± 840	15,760 ± 1,570	15,190 ± 660
0.1	11,550 ± 1,120	8,670 ± 860	3,920 ± 350	2,680 ± 690
0.01	1,370 ± 470	710 ± 250	1,481 ± 160	1,370 ± 360
0	1,150 ± 200	760 ± 150		

Table 2 *Growth-promoting activity of ascitic fluid from various patients*

HEY cells were cultured without FCS for 48 h, harvested, and plated at 1 × 10^4 cells/ml. Cells were incubated with 1% (v/v) of the indicated ascitic fluids from patients with ovarian cancer, hepatic cirrhosis, mammary adenocarcinoma, and lung carcinoma. In addition, cells were incubated with 1% (v/v) partially purified ascitic fluid (calculated on the basis of starting ascitic fluid volume), plasma, and FCS. [^{3}H]Thymidine (1 μCi/well) was added 24 h later for a 6-h pulse. Cells were harvested and counted by liquid scintillation. Results represent mean ± SEM of 4 replicates; one of 5 experiments is shown.

	[^{3}H]Thymidine incorporation (cpm)
Ascitic fluid	
Ovarian cancer	
I	12,890 ± 2,140
II	17,160 ± 680
III	17,680 ± 1,450
IV	21,680 ± 950
V	13,900 ± 1,270
VI	18,399 ± 510
VII	19,370 ± 740
VIII	18,500 ± 750
IX	17,810 ± 160
Partially purified ascitic fluid	14,680 ± 1,280
Hepatic cirrhosis	1,120 ± 500
Mammary adenocarcinoma	960 ± 500
Lung carcinoma	8,740 ± 680
Plasma	19,650 ± 1,600
FCS	13,255 ± 1,450
0	2,790 ± 200

Table 3 *Colony formation induced by ascitic fluid*

Fresh ovarian cancer cells (3–4 × 10^4/plate) or HEY cells (1 × 10^3/plate) were incubated for 15–21 days with 1% (v/v) ascitic fluid from 3 different patients or 1% FCS. Colonies were counted with an inverted microscope. Colonies were counted if they contained more than 15 cells. Each data point represents an individual experiment with at least 4 replicates. The experiment represents one of 5 performed.

	Colony frequency/(10^3 cells)		
	Medium	Ascitic fluid	Fetal calf serum
Hey cells	0	53 ± 1	58 ± 0.6
Fresh ovarian cancer cells			
I	0	6 ± 1	
II	0	4 ± 0.8	
III	0	1 ± 0.4	

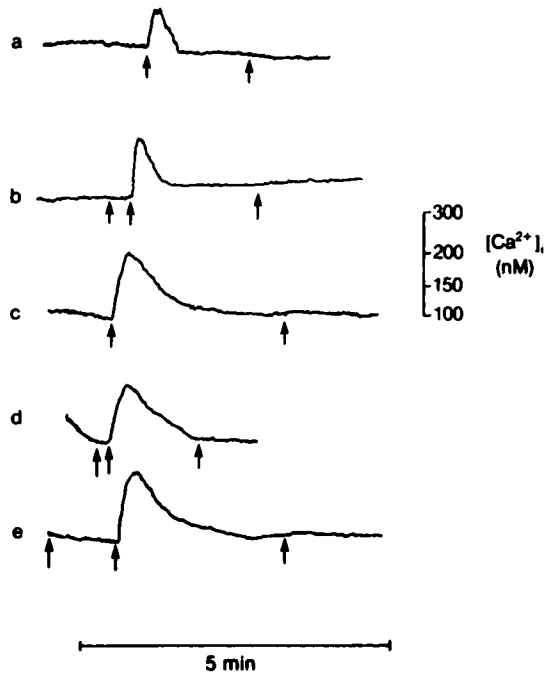

Fig. 1. Ascitic fluid increases $[Ca^{2+}]_i$ in fresh ovarian cancer cells. Fresh ovarian cancer cells (1 × 10^7/ml) were incubated with 2.5 μg/ml of the acetoxymethyl derivative of Indo-1 for 20 min in RPMI 1640. Cells were washed in RPMI 1640 and maintained at 1 × 10^6 cells/ml in RPMI 1640. Just prior to use, cells were washed in Buffer A and suspended in 1.7 ml of Buffer A with or without Ca^{2+}, as indicated, at 1 × 10^6 cells/ml in a stirred, heated cuvette. Cells were incubated until a baseline was obtained and either crude ascitic fluid (5 μl) or partially purified ascitic fluid (5 μl, calculated on the basis of starting volume of ascitic fluid) was added as indicated. For Ca^{2+}-free medium in tracings *b*, *d*, and *e*, cells were incubated until a baseline was obtained in nominally Ca^{2+}-free medium and EGTA (1 mM) was then added at the first *arrow* followed by ascitic fluid at the second *arrow*. Additions were as follows: *a*, Patient 1, Ca^{2+}-containing medium; partially purified ascitic fluid, partially purified ascitic fluid; *b*, Patient 1, Ca^{2+}-free medium; EGTA, partially purified ascitic fluid, partially purified ascitic fluid; *c*, Patient 2, Ca^{2+}-containing medium, ascitic fluid, ascitic fluid; *d*, Patient 2, Ca^{2+}-free medium; EGTA, ascitic fluid, ascitic fluid; *e*, Patient 3, Ca^{2+}-free medium; EGTA, ascitic fluid, ascitic fluid.

was lost on boiling for 3 min. At least a portion of the activity can be bound to lectin affinity columns and therefore is likely glycosylated.

Increases in $[Ca^{2+}]_i$ by Ascitic Fluid. Increases in $[Ca^{2+}]_i$ are associated with cell proliferation induced by mitogens including the polypeptide growth factors EGF; TGF-α; thrombin; and PDGF (reviewed in Refs. 14 and 15). Crude and partially purified ascitic fluid induced similar increases in $[Ca^{2+}]_i$ in fresh ovarian cancer cells (Fig. 1) and in HEY cells (Fig. 2), when the cells were loaded with the Ca^{2+}-sensitive fluorescent dye Indo-1 (9, 10). This suggests that the growth-promoting activity in ascitic fluid may activate cells through a similar mechanism to other growth factors. Base-line $[Ca^{2+}]_i$ is approximately 100 nM and it increased approximately 2–3 fold within seconds of addition of crude or partially purified ascitic fluid. Peak levels occurred within 1 to 2 min and $[Ca^{2+}]_i$ returned to baseline, or near baseline, within 5 min (Figs. 1 and 2). The concentration dependence was similar to that seen for cell proliferation with 0.1 to 1% ascitic fluid causing maximal changes in $[Ca^{2+}]_i$ (not presented). As with the proliferative response, ascitic fluid from the patient with hepatic cirrhosis and the patient with breast cancer (not presented) did not alter $[Ca^{2+}]_i$ in either HEY cells (Fig. 2*c*) or fresh ovarian cancer cells. Also similar to its effect on cell proliferation, ascitic fluid from the patient with lung cancer induced changes in $[Ca^{2+}]_i$ but was approximately 10 times less active in inducing changes in $[Ca^{2+}]_i$ than ascitic fluid from ovarian cancer patients (not presented). Therefore the two assays, proliferation and changes in $[Ca^{2+}]_i$, showed good cor-

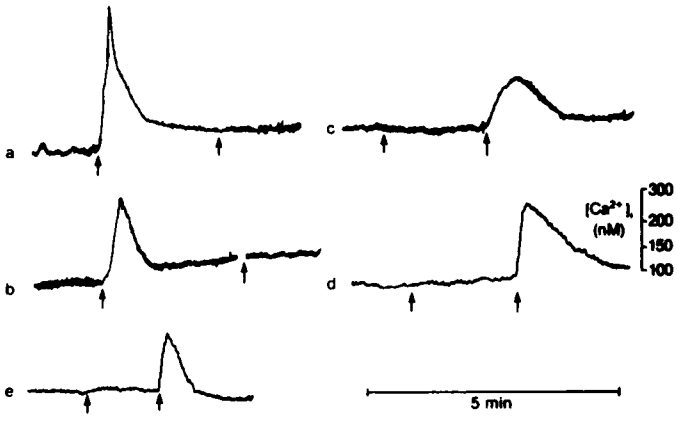

Fig. 2. Ascitic fluid increases $[Ca^{2+}]_i$ in HEY cells but not peripheral blood lymphocytes. HEY (1 × 10^7/ml) or human peripheral blood lymphocytes (1 × 10^7/ml) were incubated with 2.5 μg/ml of the acetoxymethyl derivative of Indo-1 for 20 min in RPMI 1640. Cells were washed in RPMI 1640 and maintained at 1 × 10^6 cells/ml in RPMI 1640. Just prior to use, cells were washed in Buffer A and suspended in 1.7 ml of Buffer A at 1 × 10^6 cells/ml in a stirred, heated cuvette. Cells were incubated until a baseline was obtained and either crude ascitic fluid from ovarian cancer patients (5 μl) or from a patient with hepatic cirrhosis (5 μl) or partially purified ascitic fluid (5 μl) was added as indicated. Fresh ovarian cancer cells (1 × 10^7) were washed in 200 μl of Buffer A (pH 7.23) followed by pH 3.0 citrate buffer for 30 min on ice. Supernatants were collected by centrifugation. Additions were as follows: *a*, HEY cells; ovarian ascitic fluid, ovarian ascitic fluid; *b*, HEY cells; partially purified ascitic fluid, partially purified ascitic fluid; *c*, HEY cells; ascitic fluid from patient with hepatic cirrhosis, ovarian ascitic fluid; *d*, peripheral blood lymphocytes; partially purified ascitic fluid, PHA (10 μg/ml); *e*, HEY cells, pH 7.23 supernatant (10 μl), pH 3.0 supernatant (10 μl).

relation with concentration dependence and patient source.

Ascitic fluid from all 16 patients induced changes in $[Ca^{2+}]_i$ of varying degrees in cells of 15 of 16 patients. In cells from the sixteenth patient, the changes in $[Ca^{2+}]_i$ were too low to be consistently detected. This result was not surprising since the patient had fewer than 5% ovarian cancer cells in the ascitic fluid. The remainder of the cells were of reticuloendothelial origin. Despite the lack of ovarian cancer cells in ascitic fluid from this patient, ascitic fluid induced increases in $[Ca^{2+}]_i$ in cells from other patients and in HEY cells. Therefore, in all patients in which a significant number of ovarian cancer cells were present, ascitic fluid induced changes in $[Ca^{2+}]_i$. All ascitic fluids were effective in increasing $[Ca^{2+}]_i$.

Increases in $[Ca^{2+}]_i$ can occur either as a result of release of Ca^{2+} from intracellular stores or via a net transmembrane influx of Ca^{2+} (14, 15). In the absence of extracellular-free Ca^{2+}, changes in $[Ca^{2+}]_i$ due to influx of Ca^{2+} are blocked (14, 15). When extracellular-free Ca^{2+} was decreased to less than 12 nM by the addition of 1 mM EGTA to nominally Ca^{2+}-free Buffer A, crude or partially purified ascitic fluid-induced increases in $[Ca^{2+}]_i$ were not altered (compare Fig. 1, *a* and *b*, *c*, and *d*, and note that the cells in *d* showed an apparent decrease in $[Ca^{2+}]_i$ in the presence of Ca^{2+}-free medium). This indicates that ascitic fluid increases $[Ca^{2+}]_i$ through the release of Ca^{2+} from intracellular stores. Similar results were obtained with HEY cells (not presented).

Crude or partially purified ascitic fluid did not alter the fluorescence signal in Indo-1-loaded cells which had been depleted of intracellular stores of Ca^{2+} by incubation with the cation ionophore, ionomycin, in Ca^{2+}-free medium (not presented). In addition, in cells in which Indo-1 fluorescence had been quenched by addition of 1 mM Mn^{2+} and 5 μM ionomycin, ascitic fluid did not alter fluorescence. These controls show that the changes in $[Ca^{2+}]_i$ were not due to interaction of ascitic fluid with the Indo-1 dye or to fluorescence of the ascitic fluid.

Both crude and partially purified ascitic fluid increased $[Ca^{2+}]_i$ in ovarian cancer cells but was without effect on both fresh and cultured lymphoid cells (Fig. 2*d*). The mitogenic lectin, phytohemagglutinin, increased $[Ca^{2+}]_i$ in the lymphoid cells which showed that activation pathways were intact and responsive to appropriate stimulation (Fig. 2*d*; Ref. 10). This suggests that receptors for the factor(s) are present on ovarian cancer cells but not on lymphoid cells. In contrast, short-term cultures of rat renal mesangial cells, NIH 3T3 murine fibroblasts, and fresh human breast cancer cells all responded with increases in $[Ca^{2+}]_i$ (not presented).

In many cases, hormones bound to receptors can be eluted from the receptor by incubation at low pH (16, 17). When fresh ovarian cancer cells were washed extensively in Buffer A (pH 7.23), the supernatants did not alter $[Ca^{2+}]_i$ in either fresh ovarian cancer cells (not presented) or in HEY cells (Fig. 2*e*). In contrast, when these washed cells were incubated at pH 3.0, supernatants increased $[Ca^{2+}]_i$ in fresh ovarian cancer cells and in HEY cells (Fig. 2*e*). When the acid-treated ovarian cancer cells were incubated with fresh ascitic fluid, washed extensively, and then acid-treated, the acid eluate increased $[Ca^{2+}]_i$. Supernatants from similarly treated lymphoid cells did not alter $[Ca^{2+}]_i$ in ovarian cancer cells (not presented). This suggests that there is a $[Ca^{2+}]_i$-inducing activity bound to ovarian cancer cells that has a slow dissociation rate at neutral pH and that the dissociation rate is increased at acidic pH so that the factor can be recovered in the supernatant.

Increase in Production of Phosphoinositols by Ascitic Fluid. Interaction of some growth factors with cell surface receptors activates phospholipase C which hydrolyzes membrane phosphatidylinositols to produce inositol phosphates (reviewed in Refs. 14 and 15). Production of inositol IP_3 by the hydrolysis of membrane phosphatidyl 4,5-bisphosphate has been implicated in the release of Ca^{2+} from intracellular stores in response to many growth factors (reviewed in Refs. 14 and 15). In order to determine if ascitic fluid increased $[Ca^{2+}]_i$ due to the production and action of IP_3, we measured the effect of partially purified ascitic fluid on the production of inositol phosphates in fresh ovarian cancer cells and in HEY cells (see Ref. 11 for details). Incubation of [^{3}H]inositol-loaded fresh ovarian cancer cells (Table 4) or HEY cells (not presented) with partially purified ascitic fluid resulted in a rapid increase in the production of inositol phosphates including IP_3. Increases in IP_3 were detectable within 1 min (30 sec in other experiments) of addition of ascitic fluid, which is similar to the time at which increases in $[Ca^{2+}]_i$ are detected. This is compatible with the activation of phospholipase C by activated receptors leading to the production of IP_3 and release of Ca^{2+} from intracellular stores.

Comparison with Other Growth Factors. Cell proliferation is often the result of the activation of several growth factor receptors on the surface of cells (1–3, 12). In contrast, purified growth factors in the absence of other stimuli can induce increases in $[Ca^{2+}]_i$ (14, 15). We therefore utilized changes in $[Ca^{2+}]_i$ to compare the activity of previously identified growth factors to that of ascitic fluid to determine if the latter activity is due to the presence in ascitic fluid of a previously characterized growth factor. As indicated in Figs. 1 and 2, incubation with ascitic fluid results in "homologous desensitization," that is, once a maximally active dose of ascitic fluid is added to cells, further additions of ascitic fluid do not result in an increase in $[Ca^{2+}]_i$. Incubation with either purified natural or recombinant preparations of hormones, which have been demonstrated to stimulate increases in $[Ca^{2+}]_i$ in other cell types (14, 15), including PDGF (Fig. 3*a*), FGF (Fig. 3*b*), EGF (Fig. 3*c*), TGF-β (Fig. 3*d*), and thrombin (Fig. 3*e*), did not alter $[Ca^{2+}]_i$ in either fresh ovarian cancer cells or HEY cells. In addition, other bioactive hormones including insulin, TNF-α, T-cell growth factor, interleukin-2, interleukin-1, angiotensin vasopressin, α- and β-interferons did not alter $[Ca^{2+}]_i$ in ovarian cancer cells (not presented). Each of these growth factors was determined, in this laboratory, to be active either in changing $[Ca^{2+}]_i$, inducing or inhibiting proliferation, or inducing cell differentiation with other cell types (not presented). With the exception of PDGF which was tested at 10 times maximal activity, each of the other factors was used at least 100 times that required for maximal activity on other cell types. In each case, pretreatment with the growth factors did not alter ascitic fluid-induced increases in

Table 4 *Ascitic fluid-increased production of inositol phosphates*

Fresh ovarian cancer cells were loaded with [^{3}H]inositol by overnight incubation. Cells were incubated with 15 mM LiCl to prevent inositol phosphate breakdown. Cells were then incubated with partially purified ascitic fluid for the indicated times and inositol phosphates determined by Dowex 1X8 column chromatography. The results represent the mean ± SEM of 4 determinations from 2 different experiments. The data were confirmed by separation on high-pressure liquid chromatography (1 experiment) and by incubation in the absence of LiCl (2 experiments).

	[^{3}H]Inositol (cpm)		
Time (min)	IP[a]	IP_2	IP_3
0	309 ± 41	373 ± 6	61 ± 7
1	573 ± 52	458 ± 21	119 ± 20
5	504 ± 22	522 ± 2	141 ± 15
10	528 ± 59	488 ± 76	140 ± 18

[a] IP, inositol-1-phosphate; IP_2, inositol-1-4-bisphosphate.

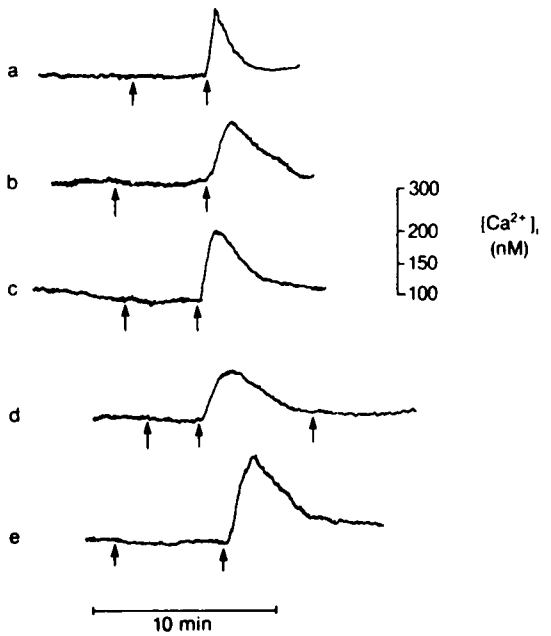

Fig. 3. Other growth factors do not increase $[Ca^{2+}]_i$. Fresh ovarian cancer cells or HEY cells (1×10^7/ml) were incubated with 2.5 μg/ml of the acetoxymethyl derivative of Indo-1 for 20 min in RPMI 1640. Cells were washed in RPMI 1640 and maintained at 1×10^6 cells/ml in RPMI 1640. Just prior to use, cells were washed in Buffer A and suspended in 1.7 ml of buffer A at 1×10^6/ml in a stirred, heated cuvette. Cells were incubated until a baseline was obtained and the indicated growth factors were added followed by partially purified ascitic fluid (5 μl). Additions were as follows: *a*, HEY cells; PDGF (5 ng/ml), partially purified ascitic fluid; *b*, HEY cells; FGF (100 ng/ml) ascitic fluid; *c*, fresh ovarian cancer cells; EGF 1 μM, ascitic fluid; *d*, fresh ovarian cancer cells; TGF-β (100 ng/ml), ascitic fluid, ascitic fluid; *e*, HEY cells, thrombin (100 ng/ml), ascitic fluid. Essentially identical tracings were obtained with TNF-α (100 ng/ml), insulin (10^{-6} M), interleukin-1 (1000 half-maximal units/ml), interleukin-2 (1000 half-maximal units/ml), vasopressin (100 ng/ml), angiotensin (100 ng/ml), and α-β or -interferon (10,000 units/ml).

$[Ca^{2+}]_i$ (Fig. 3 and not presented). Since none of these growth factors increased $[Ca^{2+}]_i$ in ovarian cancer cells and none of the growth factors desensitized the cells to the ascitic fluid factor, the factor in ascitic fluid which increases $[Ca^{2+}]_i$ in ovarian cancer cells appears to represent a unique bioactive hormone(s).

DISCUSSION

Ascitic fluid from all of the ovarian cancer patients tested ($n = 16$) contains the growth factor(s) required for the proliferation of both fresh ovarian cancer cells ($n = 5$) and the ovarian cancer cell line HEY (Tables 1–3). In contrast, ascitic fluid did not induce proliferation of lymphoid cells isolated from peripheral blood. Ascitic fluid was sufficient to induce colony formation of fresh ovarian cancer cells in semisolid methylcellulose which is considered to be a model for the proliferating "stem" cell *in vivo* (12, 13). This raises the possibility that ovarian cancer cells proliferate in the patient, at least in part, due to the action of a growth-promoting activity contained in ascitic fluid. The proliferation of ovarian cancer cells may represent an abnormal response to growth factors normally present in the patient since both plasma from normal patients and fetal calf serum contains similar growth-promoting activity (Tables 1 and 2). Because we cannot obtain sufficient nonmalignant ovarian epithelial cells to measure proliferation, we have been unable to test this possibility. Alternatively, ascitic fluid-induced proliferation of ovarian cancer cells may represent the presence of a growth factor activity which is not normally present in the peritoneal cavity since ascitic fluid from patients with hepatic disease and some nonovarian cancer malignancies did not contain similar activity.

The growth-promoting activity of ascitic fluid is associated with rapid increases in $[Ca^{2+}]_i$ presumably through receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate and the resultant production of IP_3. IP_3 in turn likely releases Ca^{2+} from intracellular stores. These changes are similar in magnitude and kinetics to the response of cells to several other growth factors and bioactive polypeptides (14, 15).

The growth-promoting activity and the activity which induces changes in $[Ca^{2+}]_i$ show good correlation. The concentration dependence and the level of activity in several different ascitic fluid preparations show a good correlation for the two assays. In addition, the two activities copurify through blue agarose and DEAE blue Sephacel.

Homologous desensitization proved to be a potent tool in demonstrating that the $[Ca^{2+}]_i$-inducing activity in ascitic fluid was different from previously characterized growth factors. Under conditions where ovarian cancer cells responded to ascitic fluid with increases in $[Ca^{2+}]_i$ EGF, PDGF, FGF, thrombin, vasopressin, angiotensin, interleukin-1, interleukin-2, interferons, TGF-β insulin, and TNF-α did not alter $[Ca^{2+}]_i$. We did not test TGF-α directly but since EGF and TGF-α bind to the same receptor (1), it is likely that TGF-α receptors would be saturated by the concentrations of EGF utilized. Similarly, although we did not test insulin-like growth factor 1, the concentration of insulin used (10^{-5} M) should bind to and saturate insulin-like growth factor 1 receptors (18). The lack of response to these bioactive factors may represent a lack of receptors for the growth factor or a lack of change in $[Ca^{2+}]_i$ in response to the growth factor or show that the receptors for the growth factors are "down regulated," removed, or inhibited by the culture or harvesting conditions. Irrespective of the cause, under conditions where ascitic fluid increased $[Ca^{2+}]_i$, none of these bioactive factors altered $[Ca^{2+}]_i$.

Although lymphoid cells do not respond to ascitic fluid with changes in $[Ca^{2+}]_i$, several other cells types did. Culture rat mesangial cells, human breast cancer cells, and murine fibroblasts responded to ascitic fluid with increases in $[Ca^{2+}]_i$ (not presented). In each case, the growth factors listed above did not block the effect of ascitic fluid on $[Ca^{2+}]_i$. Whether the same growth factor-like activity is inducing increases in $[Ca^{2+}]_i$ in ovarian cancer cells and in the other cell types will only be apparent after purification of the factor(s) to homogeneity.

Macrophages present in ascitic fluid from ovarian cancer patients have been reported to either increase (12, 20, 21) or decrease (12, 19, 21) cloning efficiency of fresh ovarian cancer cells and ovarian cancer cell lines. The relationship of the monocyte-secreted factor to the active factor(s) presented in this study is unknown at the present time. Since we have been unable to demonstrate that ovarian cancer cells themselves secrete the activity which results in changes in $[Ca^{2+}]_i$ and cell proliferation (not presented), it may be possible that this activity is also secreted by macrophage or monocytic cells. We have demonstrated that interleukin-1, a potent growth factor produced by macrophages (22), does not alter $[Ca^{2+}]_i$ in ovarian cancer cells, eliminating this factor as a possible candidate.

Ascitic fluid from mice with transplantable tumors and fresh human ascitic fluid has been shown to contain a TGF-like activity (23) which can increase the proliferation of NRK cells, the standard assay for TGF-β. Furthermore, urine from ovarian cancer patients contains high-molecular-weight TGF activity suggesting that TGF can be released by ovarian cancer cells (24). In addition, TGF activity can be eluted directly from solid ovarian cancer biopsies (25). Although the ascitic fluids studied herein likely contained TGF activity, the increases in $[Ca^{2+}]_i$

were not mimicked either by TGF-β or EGF which would be expected if the active factor were one of the TGFs.

Ascitic fluid from ovarian cancer patients has been reported to increase colony formation of the ovarian cancer cell lines H-134 and OVCAR-3 (23) and of fresh ovarian cancer cells (26). The growth factor activity could not be solely explained by the presence of EGF in ascitic fluid, but no other characterization of the active factor was made (23).

In summary, we have presented evidence that ascitic fluid from ovarian cancer patients contains a growth factor activity which is associated with rapid changes in $[Ca^{2+}]_i$ presumably as a consequence of phospholipid hydrolysis and resultant release of Ca^{2+} from intracellular stores. Increases in $[Ca^{2+}]_i$ were used to monitor the presence or absence of growth factor activity in ascitic fluid, the purification procedure, and to compare the growth factor activity to that of previously characterized factors. The growth factor-like activity appears to induce changes in $[Ca^{2+}]_i$ through binding to a specific high-affinity cell surface receptor. This factor(s) which increases $[Ca^{2+}]_i$ in ovarian cancer cells appears to be different from previously described growth factors and represents a potential new growth factor. In addition, the data suggest that ovarian cancer cells may proliferate in the cancer patient, at least in part, due to the action of a growth factor present in ascitic fluid. Furthermore, the growth-promoting activity of ascitic fluid may contribute to the propensity of ovarian cancer to remain localized to the peritoneal cavity.

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