

Acellular fraction from malignant effusions has cytotoxicity in breast cancer cells

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Abstract. Malignant ascites (MA) and malignant pleural effusion (MPE) are frequently developed in patients with metastatic cancer; however, the biological properties of these fluids have not been clarified. The present study explored the biological role of a low molecular fraction derived from malignant effusions on the activation of peripheral blood mononuclear cells and on the proliferation of breast cancer cells and fibroblast 55x cells. A <10-kDa fraction from effusions of 41 oncological patients and 34 individuals without cancer was purified, and its potential role in inhibiting nitric oxide (NO) production on lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells was explored, as well as its cytotoxicity on MCF-7 breast cancer cells and fibroblast 55x cells. A significant decrease in NO production was observed in the <10-kDa fraction from malignant effusions. In addition, the acellular fraction from MA decreased the viability of breast cancer cells without affecting human fibroblasts. These data support the presence of low molecular weight molecules in malignant samples with a specific role in inhibiting the

defense mechanisms of peripheral blood mononuclear cells and decreasing the viability of breast cancer cells *in vitro*.

Introduction

Malignant ascites (MA) or malignant pleural effusions (MPE) are a common clinical manifestation in patients with advanced neoplasia and confer a poor prognosis (1,2). It is known that MA and MPE stimulate an aggressive cellular phenotype and generate a pro-inflammatory environment that promotes immunosuppression and allows the proliferation and dissemination of cancer cells (3-5). Growth factors, cytokines, and glycoproteins have been found to have higher concentrations in MA and MPE than in plasma (6-9). Such biomolecules include vascular endothelial growth factor, angiogenin, epidermal growth factor, interleukin-6, monocyte chemoattractant protein-1, transforming growth factor beta-1, and secreted phosphoprotein-1 (10-12). All of these molecules play an important role in tumor growth, angiogenesis, and metastasis, which shorten the survival of patients with cancer. Other studies have found elevated levels of several proteases in malignant effusions (13,14). Our group previously reported a macrophage-activation inhibitory factor (MAIF), which was purified from mouse ascites by L5178Y murine lymphoma cells and inhibited lipopolysaccharide (LPS)-induced macrophage activation (15). MAIF also allowed the development of hepatic abscesses *in vivo* when BALB/c mice were inoculated with *Entamoeba histolytica* or *Listeria monocytogenes* (16,17).

Nevertheless, some reports have demonstrated the anti-tumor role of MA and MPE. Cohen *et al* described the pro-apoptotic effect of cell-free ascites by activation of the JNK pathway and induction of BRCA1, Fas, and FasL expression in SKOV3 cells (18). Other studies have shown the

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existence of angiogenesis and migration inhibitors in ascites and pleural effusion from patients with breast cancer, ovarian carcinoma, lung carcinoma, and mesothelioma (19-22). These findings indicate that the biochemical compositions of MA and MPE are widely diverse and that these effusions can play dual roles in tumor progression.

Macrophage activation by LPS polarizes them to the M1 phenotype and can produce nitrogen-based radicals by stimulating inducible nitric oxide synthase (iNOS) (23-25). Thus, increased nitric oxide (NO) production can reflect polarization to a proinflammatory phenotype.

The present study sought to explore whether the MA-MPE-derived acellular fraction could modulate the production of NO by peripheral blood mononuclear cells (PBMCs) and whether NO influences the viability of healthy and cancerous cells.

Materials and methods

Clinical specimens. Forty-one malignant effusion samples were collected from patients diagnosed with primary neoplasia and 34 samples were derived from patients with non-cancer diagnoses. All samples were obtained at Instituto Mexicano del Seguro Social, in Monterrey, Mexico. The study was approved by the Institutional Ethics Board with the registration number R-2008-1908-2, and written informed consent was obtained from each patient before participation. Patients with thrombocytopenia, abnormal clotting time, HIV/AIDS, or primary immunodeficiency diseases were excluded.

Collection of biological samples. The pleural effusion and ascitic fluids used in this study were collected by thoracentesis or paracentesis, respectively, at the time of the therapeutic protocol. Approximately 20 ml was taken for each specimen under aseptic conditions. All samples were stored at -20°C until analysis.

Purification of the <10 kDa fraction. To guarantee the exclusive presence of low-molecular-weight biomolecules, all samples were depleted of cells by centrifugation at 30,000 g for 20 min, and each cell-free supernatant was purified using centrifugal filter units with membranes having a nominal molecular weight cutoff of 10 kDa (Merck Millipore). The <10 kDa fraction was aliquoted into 1 ml vials, and protein concentration was determined using the Lowry test. The samples were stored at -20°C until analysis.

Stimulation of peripheral whole blood. To analyze the production of NO by PBMCs, the whole blood of a healthy volunteer was recollected into plastic blood collection tubes with sodium citrate (Becton, Dickinson and Company). Aliquots (3 ml) were made within the first 60 min of blood collection and were then stimulated with 30 µg/ml of the <10 kDa fraction at 37°C with 5% CO₂ for 2 h, with constant agitation. Subsequently, without removing the <10 kDa fraction, each sample was treated with 50 ng/ml *E. coli* serotype O12B:B12 LPS (Sigma-Aldrich; Merck KGaA) and incubated for 5 h under the conditions previously described. After that, we obtained the plasma by centrifugation at 2,000 g for 10 min and stored the samples at -80°C until analysis. In addition, the three control groups

were shaped: a) a group with LPS-unstimulated blood, b) an LPS-treated group as positive control, and c) an LPS-treated group treated with 100 ng/ml of NG-monomethyl-L-arginine acetate (Sigma-Aldrich; Merck KGaA) as NO inhibitor.

Nitric oxide assay. NO concentration was measured using the total nitric oxide assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, to convert nitrate to nitrite, 50 µl of plasma was plated in 96-well plates with the nitrate reductase enzyme for 30 min at 37°C. Nitrite was detected as a colored azo dye product at 540 nm in a microplate reader (BioTek Instruments, Inc.), and the results were expressed in micrometers.

Cell lines and cell culture. Human lung fibroblast 55x and MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC). They were cultured at 37°C with 5% CO₂ in DMEM supplemented with 10% heat-inactivated fetal bovine serum Gibco™ (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich; Merck KGaA). The medium was changed every three days, and the cells were passaged twice weekly.

Cytotoxicity assay. Cell growth inhibition was measured using the MTT assay (Abcam) at 24 h post exposure. Briefly, 1x10⁴ cells were seeded into 96-well culture plates and cultured for 24 h. After exposure to each sample at 2% v/v, cells were washed twice with phosphate-buffered saline (Gibco™; Thermo Fisher Scientific, Inc.) twice, and 100 µl of MTT solution (5 mg/ml in medium) was added to each well. Then, the formazan in viable cells was dissolved in acidified isopropanol solution and measured at 570 nm using a microplate reader Elx 800 (BioTek Instruments, Inc.). The absorbance value of cells incubated with culture medium (untreated group) was set to 100% cell viability and compared with treated cells. We used 1% Triton X-100™ (Sigma-Aldrich; Merck KGaA) and vincristine (500 µg/ml) as the cytotoxic control.

Statistical analysis. Each experimental protocol was tested in triplicate and repeated three times in independent experiments, and the average was used for the analysis. Data are expressed as mean and standard deviation. Student's t-test or Fischer's exact test were used to compare the characteristics of patients with MA and malignant pleural effusion. One-way ANOVA with Tukey's post hoc test was used for comparisons among multiple groups. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Subjects. The clinical characteristics of the patients with cancer are shown in Table I. Twenty-one ascite samples from patients with primary tumor diagnoses and 20 samples of malignant pleural effusion were examined. The majority of patients were categorized as stage IV at the time of sample collection. In patients with MA, the more frequent metastatic sites were the peritoneum (13/21) and liver (5/21), followed by the lungs (2/21) and spleen (1/21), while all MPEs were obtained from patients with thoracic metastases. Benign ascites (BA)

Table I. Clinical characteristics of oncological patients.

Characteristic	Malignant ascites	Malignant pleural effusion	P-value
Age, years			
Mean \pm SD	51.33 \pm 12.09	67.45 \pm 14.86	0.004 ^a
Range	35-75	21-87	
Sex, n (%)			
Male	7 (33.3)	10 (50.0)	0.279 ^b
Female	14 (66.6)	10 (50.0)	
Diagnosis (n)	Ovarian cancer (6), lung cancer (1), hepatocellular carcinoma (3), lymphoma (2), breast cancer (1), mesothelioma (2), melanoma (1), gastric cancer (1), cancer of unknown primary (3), pancreatic cancer (1)	Ovarian cancer (1), lung cancer (10), bone and soft tissue tumors (3), lymphoma (1), breast cancer (3), mesothelioma (1), renal cell carcinoma (1)	
Clinical stage, n (%)			
III	3 (14.2)	1 (5.0)	0.317 ^b
IV	18 (85.7)	19 (95.0)	
ECOG score, n (%)			
2	0 (0.0)	12 (60.0)	
3	15 (71.4)	5 (25.0)	0.003 ^b
4	6 (28.5)	2 (10.0)	0.134 ^b
5	0 (0.0)	1 (5.0)	
Treatment, n (%)			
Chemotherapy	13 (61.9)	4 (20.0)	0.006 ^b
Radiotherapy		0 (0.0)	4 (20.0)
Both		1 (4.7)	0 (0.0)
None	7 (33.3)	12 (60.0)	0.087 ^b

^aStudent's t-test; ^bFischer's test. ECOG, Eastern Cooperative Oncology Group.

samples were collected from 18 cirrhotic patients, of which 16 patients were male. We obtained benign pleural effusion (BPE) from patients with congestive heart failure (n=6), chronic kidney disease (n=4), pneumothorax (n=1), pancreatitis (n=2), panlobular emphysema (n=1), rib fracture (n=1), and penetrating abdominal trauma (n=1).

Patients with MA were younger than patients with malignant pleural effusions (51.33 \pm 12.09 vs. 67.45 \pm 14.86; P<0.01). There were no differences between the proportion of male/female samples or clinical stage among patients with MA and malignant pleural effusions. However, MA samples were more frequent from patients with a history of chemotherapy or with a ECOG grade 3 (Eastern Cooperative Oncology Group scale) (Table I).

NO production. In the LPS-stimulated group, NO production was twice as high as in the inhibitor group (102.2 \pm 15.50 vs. 58.6 \pm 10.41 μ M; P<0.001). Similarly, the amounts of NO differed between benign (91.87 \pm 10.97 μ M; P<0.001) and malignant (62.06 \pm 15.63 μ M) ascites samples, and also BPE and MPE samples differed (Fig. 1).

MA and MPE modulated cytotoxicity in breast cancer cells. A cell viability assessment was performed on some samples

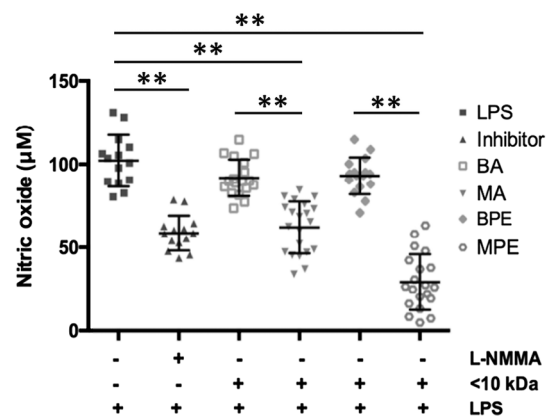


Figure 1. Nitric oxide production by peripheral blood mononuclear cells incubated with the <10-kDa fraction of ascitic or pleural effusions and stimulated with *Escherichia coli* LPS. Data were analyzed with ANOVA followed by Tukey's post hoc test. **P<0.01. LPS, lipopolysaccharide; BA, benign ascites; MA, malignant ascites; BPE, benign pleural effusion; MPE, malignant pleural effusion; L-NMMA, NG-Monomethyl-L-arginine acetate.

from MA (n=12), BA (n=10), MPE (n=8), and BPE (n=8). MA samples induced reduction of MCF-7 cell viability in comparison with BA (55.82 \pm 16.11 vs. 78.47 \pm 21.52; P<0.01); also, the

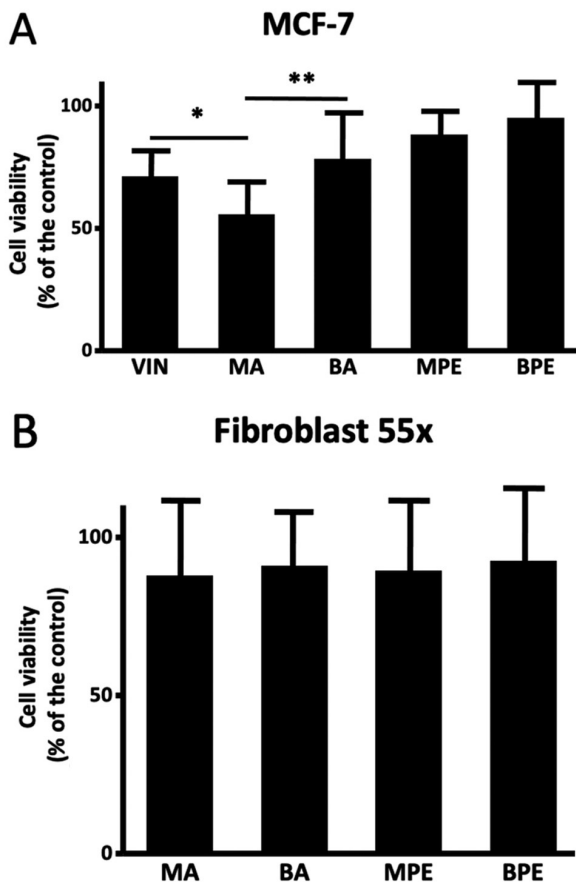


Figure 2. Viability of (A) MCF-7 and (B) fibroblast 55x cells incubated with a <10-kDa fraction of ascitic or pleural effusions. Data were analyzed with ANOVA followed by Tukey's post hoc test. * $P < 0.05$; ** $P < 0.01$. BA, benign ascites; MA, malignant ascites; BPE, benign pleural effusion; MPE, malignant pleural effusion.

cytotoxic effect of MA was higher than that of vincristine (71.20 ± 13.67 ; $P < 0.05$), and there was no difference with MPE or BPE (88.36 ± 11.05 and 95.15 ± 14.31) (Fig. 2A). None of the samples, either malignant or benign, affected the viability of Fibroblast 55x cells (Fig. 2B).

Discussion

In this study, we evaluated the NO production by PBMCs exposed to an acellular fraction derived from MA/MPE. Our results demonstrated that the acellular fraction of MA/MPE can reduce NO production in PBMCs stimulated with LPS. We also determined that the addition of MA/MPE decreased cancer cell viability *in vitro*, but did not affect healthy fibroblasts.

MA effusions are created by the tumor and act as a unique environment that is dominated by tumor-induced interactions. They provide a framework that orchestrates cellular and molecular changes that contribute to aggressiveness and disease progression (26,27). These effusions are rich in cytokines, chemokines, growth factors, and immune effector cells (25-27); however, their antitumor functions have been reported to be negatively regulated (27). Our results are in accordance with this finding, and the NO production in LPS-stimulated macrophages decreased when they were incubated with the <10 kDa fraction. This macrophage

activation failure contributes to the survival of tumor cells despite the proinflammatory environment. This is supported by our previous observations that MA derived from L5178Y murine lymphoma fails to activate macrophages when the cells are pre-treated with cell-free MA before stimulation with LPS (14). However, there is evidence that macrophages exposed to different environments can change their polarization, and perhaps the phenotypic change from M1 to M2 could explain the lower production of NO when the PBMCs were pre-incubated with malignant effusion extracts (23-25).

There is evidence that NO has a dual role, where a low NO concentration inhibits proliferation in some tissues while in others it inhibits apoptosis, and its effects are dose-, cell-, and even cancer stage-dependent (28-31), we observed a decrease in the viability of tumor cells that could be related to the decrease in NO.

Unlike MA, in patients with cancer, pleural effusions can develop as a result of the interference with the integrity of the lymphatic system, direct tumor involvement of the pleura, and local inflammatory changes in response to tumor invasion (32). Furthermore, like MA, the presence of cancer cells in pleural effusion defines MPE. Soini *et al* (33) reported higher NO production by iNOS in MPEs than in benign ones. Our MPE samples inhibited macrophage NO release in a similar way as MA samples, but its effect on cancer cell survival was less evident.

Although some studies have shown the heterogeneity of the soluble components in the malignant fluid (34-36) and heterogeneity in the type of cancer that produced our samples, the decrease in cancer cell viability upon incubation with the <10 kDa fraction and its innocuity in healthy cells, reveals the presence of a common anti-tumorigenic molecule in all malignant effusions. According to our data, we can speculate that the <10 kDa fractions derived from MA and MPE contain biological molecules that modulate the activation of PBMCs and regulate breast cancer proliferation. The next step is to profile the biochemical composition of the <10 kDa fractions derived from malignant fluids.

Limitations. All blood samples came from the same subject; however, we recognize that plasma protein concentration before or after stimulation was not considered and could affect the cytotoxicity assay. It is also worth considering that we did not perform a cytotoxicity assay on PBMC, nor did we evaluate the cytotoxicity of the PBMC-stimulated extract. We only performed a cytotoxicity assay using the <10 kDa fraction.

In conclusion, independent of cellular origin, low molecular weight fractions derived from MA and MPE had molecules that inhibited PBMC defense mechanisms and decreased the viability of breast cancer cells *in vitro*.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JVV, MCR and RPC conceived the original research idea and developed the experimental design. AEO, HGH and EDDLJ designed the study. AEO, HGH, EDDLJ, OMD, CAMA, FGS and MGMT performed the experiments and acquired the data. FJGDLG, JVJ, MCR and RPC conducted data analysis and revised the manuscript. FJGDLG, JVJ and MCR were responsible for interpretation of data and manuscript writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the National Committee for Scientific Research of Instituto Mexicano del Seguro Social (Mexico City, Mexico; registration no. R-2008-1908-2). Written informed consent was obtained from all patients before their participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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