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Spontaneous Release of Interleukin-6 by Primary Cultures of Lymphoid and Tumor Cell Populations Purified from Human Ovarian Carcinoma

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ABSTRACT

Interleukin-6 (IL-6) is a cytokine that has been implicated as a growth factor in human ovarian carcinoma, yet the *in vivo* source of IL-6 in patients remains undefined. We measured IL-6 by ELISA in cell-free ascites (CFA) of 19 patients with ovarian carcinoma. IL-6 was detectable in all samples (mean level 3.3 ng/ml). To identify the cellular source of IL-6, we measured this cytokine by ELISA in 24–48 h supernatants of cultured lymphocyte-, macrophage-, and tumor cell-enriched populations purified from three solid ovarian carcinomas by centrifugal elutriation. All cell populations spontaneously released IL-6; however, tumor cells and tumor-associated macrophage released levels of IL-6 that greatly exceeded those released by tumor-associated lymphocytes. Kinetic studies revealed that IL-6 was detectable at 6 h and that levels increased in all cultures examined over a 48 h time course. These data suggest that both tumor and infiltrating host cells may be the source of the high levels of IL-6 found in carcinomatous ascites. Furthermore, although all three cell types examined may contribute to IL-6 production in patients with ovarian carcinoma, tumor cells are perhaps the most clinically significant source.

INTRODUCTION

OVARIAN CARCINOMA is the fourth most frequent cause of cancer death in women in the United States. Because of the disease's insidious onset and progression, 65–75% of patients initially present with tumor disseminated throughout the peritoneal cavity.[1] Although the interaction between host and tumor cells is complex, most investigators believe that cytokines and their naturally occurring antagonists act locally in the tumor microenvironment and regionally in the peritoneal cavity to regulate tumor cell growth as well as to orchestrate cell-mediated antitumor activity.[1–7] Nevertheless, the cellular origins of cytokines and their natural inhibitors, as well as the specific mechanisms by which cytokines and cytokine inhibitors function in the control and pathogenesis of this disease, are poorly understood.

In general, interleukin-6 (IL-6) is a pleiotropic cytokine secreted by a wide variety of cell types, including lymphocytes, monocytes, and tumor cells.[8] The biologic effects of IL-6 on human epithelial tumors *in vitro* are diverse; however, these effects appear to be tumor type specific. Results from other laboratories suggest that IL-6 can act as an autocrine or paracrine growth factor in human colon,[9] renal cell,[10] and cervical[11] carcinoma *in vitro*. In contrast, IL-6 has been shown to inhibit the growth of human non-small cell lung cancer[12] and breast cancer[13] cells *in vitro*. Other investigators have observed additional tumor-promoting and antitumor effects of IL-6.[14–19] Studies on human breast carcinoma cells *in vitro*, in particular, show that IL-6 protects these tumor cells from natural killer-mediated killing,[14] decreases adherence to neighboring tumor cells,[20] and increases tumor cell motility.[15,16] Antitumor effects may also be exerted indirectly, either through stimulation of cytotoxic T cells[17] or through boosting the immunogenicity of tumor cells by upregulating the expression of cell surface antigens.[18,19]

Conflicting *in vitro* data exist regarding the role of IL-6 in human ovarian carcinoma. IL-6 has been detected by enzyme-linked immunosorbent assay (ELISA) in the serum[21] and ma-

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lignant ascites[22] of ovarian cancer patients, as well as in super-
natants of continuous human ovarian carcinoma cell lines[22]
and cultured ovarian tumor-associated macrophages.[23] We
were interested in determining whether we could identify a
cellular source for IL-6 detected in the ascites of women with
ovarian carcinoma. To do so, we examined the spontaneous
release of IL-6 in vitro from short-term cultures of purified
lymphocytes, macrophages, and tumor cells derived from fresh
solid ovarian tumors.

MATERIALS AND METHODS

Patients and samples

Samples of malignant ascites were obtained from 19 patients
with advanced ovarian carcinoma at the time of diagnostic and
staging laparotomy and clarified by centrifugation. Samples of
solid malignant ovarian carcinoma (E1–E3) were obtained from
the peritoneal cavities of three additional patients, all with stage
III adenocarcinoma, at the time of diagnostic and staging lap-
arotomy. These tumor samples were then placed immediately in
sterile complete media consisting of RPMI 1640, 15% fetal calf
serum, and 1% penicillin and streptomycin and 1% L-glutamine
(GIBCO, Grand Island, NY) in lipopolysaccharide-free sterile
water.

Tumor processing

Solid tumor samples were processed under sterile conditions
at room temperature as described by Weisenthal and col-
leagues.[24] Briefly, grossly viable tumor was mechanically
minced in RPMI 1640 and enzymatically dissociated with
0.14% collagenase type I (Sigma) and 0.01% DNase (Sigma,
2000 kunits/mg) in RPMI 1640 overnight at 4°C. Enzymatically
dissociated tumor was then filtered through 150 μm nylon mesh
to generate a single-cell suspension. The resultant cell suspen-
sion was then washed twice in complete media.

Centrifugal elutriation

Cell separation was performed utilizing a JE-6B elutriation
system and rotor with a Sanderson-type chamber in a J2-21
centrifuge (Beckman Instruments, Inc.). Elutriation was per-
formed according to a protocol adopted from the technique of
Moore and Mortari.[25] Briefly, with rotor speed calibrated to
4000 rpm, a suspension containing 10^8 cells in 20 ml elutriation
buffer was infused at a constant flow rate of 30 ml/minute. For
the first two tumors processed (E1 and E2), the rotor speed was
decreased in a stepwise fashion in 100 rpm increments to a final
speed of 1600 rpm, and at each speed, 100 ml eluate was
collected. Based on the characteristics of fractions obtained at
each centrifuge speed in the first two trials, the protocol was
simplified for the third tumor processed (E3), in which 500 ml
fractions were obtained at 4000, 3600, 3200, 2800, 2000, and
1600 rpm. Any cells retained in the Sanderson chamber were
transferred to a sterile 50 ml polypropylene tube (Dow Corning)
containing elutriation buffer. Cell suspensions were centrifuged
and resuspended in complete media.

Cell counts and viability determination

Cell count and viability by trypan blue exclusion were deter-
mined for the original cell suspension as well as for each elutri-
ated fraction, before cell culture. There was no loss of viability
for the elutriation procedure. The initial mean percentage via-
bility for elutriated fractions ranged from 79 to 97%. The mean
percentage decrease in viability after 24 h culture for elutriated
fractions of these tumors was 41% (E1), 10% (E2), and 1.3%
(E3).

Cell morphology characterization

Morphology was characterized for the original cell suspen-
sion as well as for each elutriated fraction by light microscopy
of cytospin preparations stained with Wright-Giemsa or hema-
toxylin-eosin. For all three tumors, morphologic characteriza-
tion was performed independently and in a blinded fashion by a
board-certified pathologist.

Cell culture

After centrifugal elutriation and morphologic characteriza-
tion, cells from both unseparated preparations and elutriated
cell suspensions were cultured at 37°C, 5% CO_2, in 1–2 ml
complete media at a density of 1×10^6 cells/ml in 3 ml poly-
propylene culture tubes. For kinetic studies, involving E3, indi-
vidual cultures were incubated for predefined periods, after
which homogeneous cell suspensions were examined for viabil-
ity and centrifuged at 1500 rpm for 10 minutes. Cell-free super-
natants were aspirated and stored at −20°C.

ELISA for IL-6

Supernatants from 24 h cultures were collected for E1–E3
and from 6, 12, 24, and 48 h cultures for E3 only. Supernatants
collected from cultures derived from E3 were tested both in
undiluted form and in a form diluted 1:100 with complete me-
dia. Concentrations of IL-6 were determined by ELISA (Re-
search & Diagnostic Systems). All samples were assayed in
duplicate, along with known standards diluted in complete me-
dia, and chromogenicity was determined by spectrophotometric
determination of optical density at the appropriate wavelength
using a EAR 400 AT plate reader (SLT Labinstruments). Stan-
dard regression lines, generated by plotting log concentration
versus log optical density, projected correlation coefficients
greater than 0.98 in all cases. The maximal allowed sample
duplicate error was 10%.

RESULTS

Detection and measurement of IL-6 in malignant ovarian carcinomatous ascites

Cell-free ascites (CFA) samples derived from 19 patients
with histologically proven advanced (stage III and IV) ovarian
carcinoma at the time of original diagnosis were analyzed for
the presence of IL-6 by ELISA. The mean concentration of IL-6
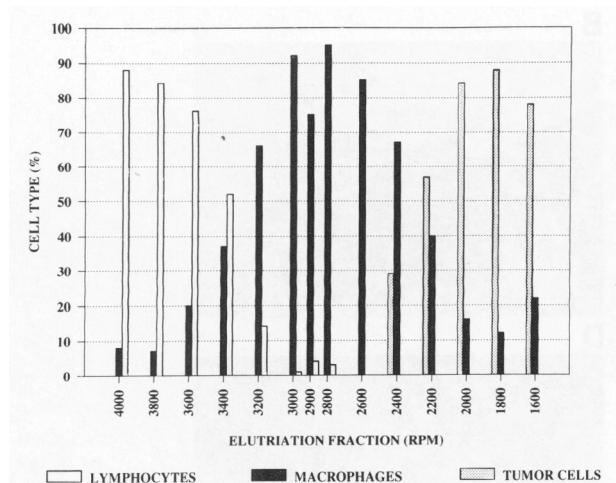in these 19 samples was 3.3 ng/ml, range 0.4–20.4 ng/ml.

FIG. 1. Elutriation profile for E1. A single-cell suspension derived from E1 was fractionated by centrifugal elutriation, as described in Materials and Methods. As rotor speed was decreased, cells of progressively increasing size were eluted. The morphology of elutriated cell suspensions was determined by microscopic evaluation of cytopreps stained with Wright-Giemsa and hematoxylin-eosin and is graphed here as the percentage of each major cell type (lymphocytes, macrophages, and tumor cells) found in elutriated cell suspensions isolated at specific rotor speeds (rpm).

Separation and characterization of cell populations derived from solid malignant ovarian carcinoma by centrifugal elutriation

Solid tumor samples, E1–E3, were obtained from three additional patients with stage IIIC serous papillary adenocarcinoma of the ovary, grades 3 (E1 and E2) and 2 (E3). Single-cell suspensions derived from these solid tumors were produced and then fractionated by centrifugal elutriation, as described in Materials and Methods. A representative elutriation profile for E1 is depicted schematically in Fig. 1, and photomicrographs of a histologic section and cytopreps from preelutriated and most uniform elutriated fractions from the same tumor are presented in Fig. 2. For E1-E3, the purity of most homogeneous elutriation fractions ranged from 84 to 99% for lymphocytes, from 75 to 92% for macrophages, and from 88 to 95% for tumor cells.

Spontaneous release of IL-6 by purified tumor-derived cell populations in short-term culture

The purest elutriated cell suspensions with respect to cell type were cultured as described in Materials and Methods, and at predefined incubation times, supernatants were retrieved for analysis by ELISA. First, levels of spontaneously released IL-6 detected in 24 h culture supernatants were determined. For tumors E1 and E2, ELISA was performed on undiluted supernatants. In these cases, supernatants of both macrophage and tumor cell cultures contained levels of IL-6 that exceeded 300 pg/ml (exact levels could not be determined because they exceeded the highest standard concentration in the assay, and no additional supernatant was available for testing); supernatants of lymphocyte cultures derived from these tumors contained only 69 and 51 pg/ml, respectively. For tumor E3, ELISA was

performed on both undiluted and 1:100 diluted supernatants, as described in Materials and Methods. In this case, as depicted in Fig. 3, the tumor cell fraction released the highest level of IL-6 (5433 pg/ml), the macrophage fraction an intermediate level (2473 pg/ml), and the lymphocyte fraction the lowest level (368 pg/ml). The kinetic pattern of spontaneous IL-6 release was then examined for tumor E3. As shown in Fig. 4, IL-6 was first detectable at 6 h in supernatants from all cultures and, over a 48 h time course, accumulated in these supernatants, 3255–6920 pg/ml for the tumor cell-rich culture, 1500–3065 pg/ml for the macrophage-rich culture, and 130–478 pg/ml for the lymphocyte-rich culture.

DISCUSSION

We detected IL-6 ranging from 0.4 to 20.4 ng/ml by ELISA in the CFA of 19 patients with advanced ovarian adenocarcinoma at the time of diagnosis. This is consistent with the work of other investigators,[26,27] who in addition demonstrated significantly lower levels of IL-6 in benign peritoneal fluids. We also noted considerable variability in ascitic IL-6 levels found among patients with advanced disease. Berek and colleagues recently showed that serum levels of IL-6 at the time of diagnosis were directly related to tumor burden and inversely related to survival.[21] Serum levels of IL-6 have been shown to be a prognostic factor in other tumors, such as renal cell carcinoma.[28]

We were interested in identifying the cellular source of IL-6 production in the peritoneal cavity of patients with ovarian carcinoma. Specifically, we asked whether IL-6 was released from specific cell types found in solid ovarian tumors. We examined the spontaneous release of IL-6 from short-term cultures of purified lymphocytes, macrophages, and tumor cells derived from solid ovarian carcinoma tumor nodules. Using a modification of the elutriation technique of Moore and Mortari,[25] we generated purified suspensions of lymphocytes, macrophages, and tumor cells from three solid ovarian adenocarcinomas. Examination of these suspensions showed minimal cross-contamination and good cell viability.

Our results show that both tumor cells and tumor-associated macrophages derived from the same solid tumors spontaneously and simultaneously released high levels of IL-6. Surprisingly, lymphocytes derived from the same tumors released considerably low levels of IL-6. Our finding of spontaneous IL-6 released by ovarian carcinoma-associated macrophages and tumor cells is supported by the work of other investigators. Watson et al. demonstrated constitutive IL-6 production by human ovarian carcinoma cell lines as well as unseparated primary ovarian carcinoma cultures.[22] Erroi et al. observed IL-6 release from macrophages purified from both ovarian carcinomatous ascites and solid tumor by plastic adherence.[23] Our kinetic studies demonstrated the appearance of IL-6 in all cultures by 6 h, with accumulation over a 48 h time course. Tumor cells demonstrated the most dramatic increase in IL-6 levels. These data suggest that IL-6 release from cells in solid tumor, principally tumor cells and tumor-associated macrophages, may be responsible for the high levels of IL-6 detected in the ascites of patients with ovarian carcinoma. In particular, tumor cells ap-
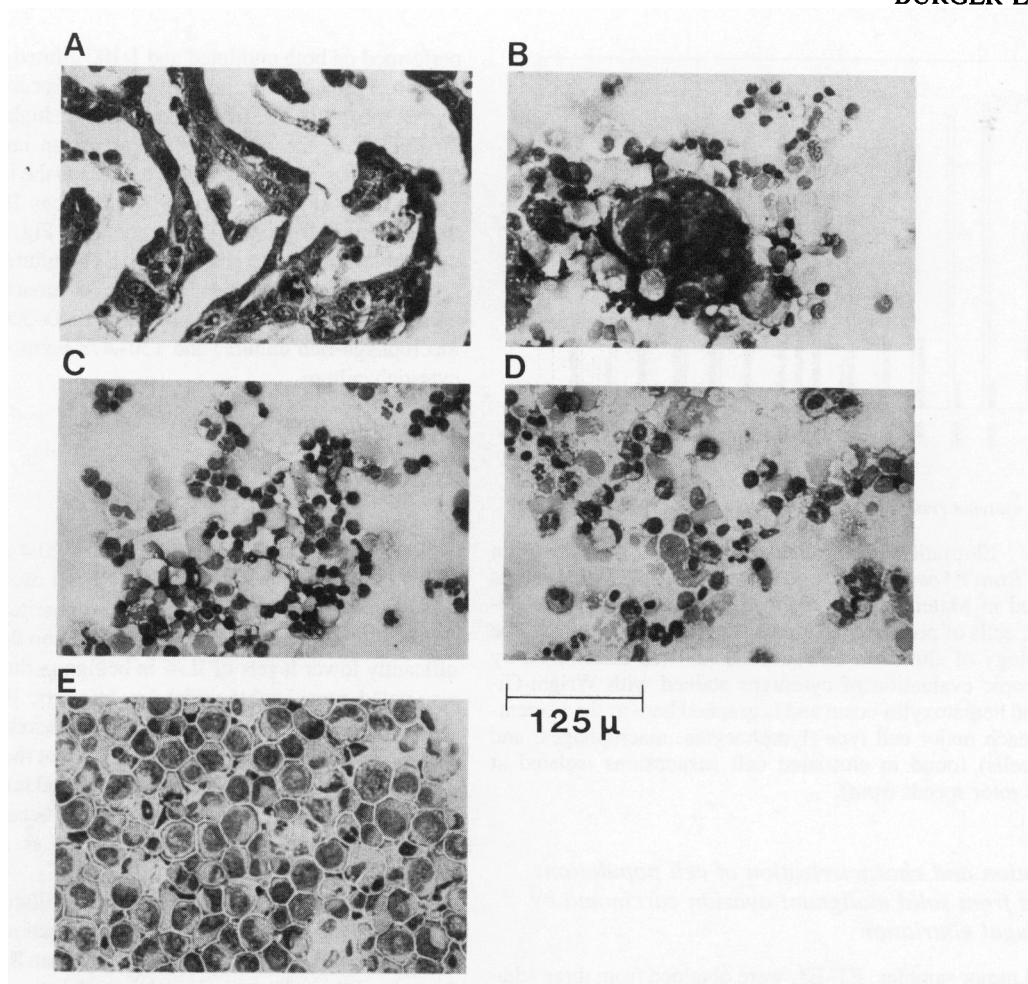
FIG. 2. Morphology of elutriated cell suspensions. The morphology of preelutriated and elutriated cell suspensions for E1 was ascertained by microscopic evaluation of cytopreps stained with Wright-Giemsa and hematoxylin-eosin. Photomicrographs are depicted in the top panels. (A) Histologic section of formalin-fixed, paraffin-embedded solid tumor. (B) Preelutriated cell suspension. (C) Lymphocyte-rich cell suspension. (D) Macrophage-rich cell suspension. (E) Tumor cell-rich cell suspension. Original magnifications: ×400.

pear to be the major source of this cytokine. This is supported by the work of Berek and colleagues, who showed that serum IL-6 levels were associated with tumor burden.[21]

There are several possible explanations for the spontaneous release of IL-6 from cells within solid tumor. Hypothetically, spontaneous and sustained release of IL-6 from macrophages and lymphocytes reflects a response (although obviously ineffective) against the tumor. Because we and others have demonstrated that IL-6 can be released from ovarian carcinoma cells, the question arises whether IL-6 may be an autocrine or paracrine growth factor in this disease, as has been demonstrated for other tumor types.[29] Wu et al. demonstrated that supernatants of cultured monocytes contained soluble factors that could stimulate the growth of certain ovarian carcinoma cell lines.[3] Recombinant IL-6 could also cause growth stimulation of these same cell lines, although the growth stimulatory effect found in monocyte supernatants was only partially neutralized by antibody to IL-6. This implied a paracrine growth effect for IL-6 in this disease. An autocrine growth mechanism was suggested by Watson et al., who demonstrated growth inhibition of IL-6-
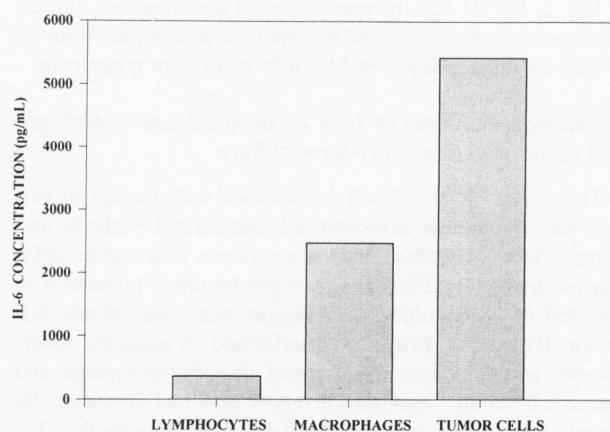

FIG. 3. Levels of spontaneously released IL-6 in 24 h supernatants. Cell suspensions derived from tumor E3 by centrifugal elutriation were cultured for 24 h, after which supernatants were obtained for measurement of IL-6 levels by ELISA.
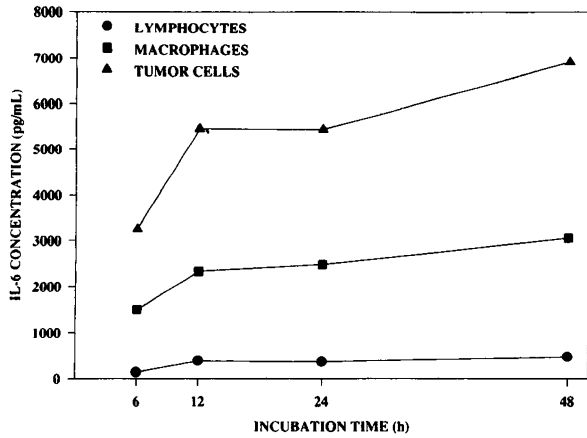
FIG. 4. Kinetics of spontaneously released IL-6 in supernatants from lymphocyte-rich, macrophage-rich, and tumor cell-rich cultures derived from E3 by centrifugal elutriation. Elutriated cell suspensions were cultured for variable incubation times, after which supernatants were obtained for measurement of IL-6 by ELISA.

producing ovarian cancer cells transfected with antisense IL-6 DNA.[30] This is particularly interesting, because it has been found that IL-6 is released by normal surface ovarian epithelial cells,[4,31] thus raising the possibility of its role in the pathogenesis of the disease.

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