Interactions between tumor cells and tumor infiltrating lymphocytes in human ovarian carcinoma

B. DONNERSTAG (*) - W. SCHRÖDER (**) - P. HOOS (*)
J. B. OLTROGGE (*) - L. TRÄGER (*) - H. G. BENDER (**)

Summary: The aim of our study was to investigate the lymphocytic infiltration rate of ovarian tumors and the possible use of tumor-infiltrating lymphocytes (TIL) as a therapeutic tool in gynecologic oncology. Twelve tumors were treated with digesting enzymes in order to isolate tumor-infiltrating lymphocytes as well as tumor cells, TIL were expanded by culture in the presence of human interleukin-2 (IL-2). Freshly prepared tumor cells were allowed to grow in culture medium for several days before the first investigations were performed. TIL could only be isolated from 50% of the investigated tumors. In contrast to this the isolated tumor cells could be largely expanded in 73% of the cases. The expression of the CA 125 antigen in the culture supernatants served as control and could still be evaluated up to three months after isolation. In parallel the antigen expression on the cellular surface was estimated by immunocytochemistry. Evaluating the phenotypes of TIL showed predominantly CD3+, their expansion rate was only poor. Tumor cells were isolated and expanded in order to test the tumor-directed cytotoxic efficacy of TIL and for further use in transplantation to nude mice.

Key words: Ovarian cancer; Tumor-infiltrating lymphocytes; Interleukin-2.

INTRODUCTION

Ovarian cancer is the 5th most common form of cancer in women [1].

Even though this tumor type is sensitive to chemotherapy, drug resistance frequently develops [2]. For studying the biology of ovarian cancer, reproducible sources of material are required. We have established a series of cell lines from 12 ovarian tumors. At the same time mononuclear cell infiltration within the tumors was examined. As the presence of immunocompetent cells within tumor tissue is purposeful rather than circumstantial, TIL have long been a subject of interest to tumor immunologists [3]. Studies of freshly isolated TIL from human tumors demonstrated that they showed poor cytotoxic activity against autologous or allogeneic tumor cells [4] and reduced ability to proliferate in response to mitogens and alloantigens [5, 6]. It was hypothesized

(*) Department of Biological Chemistry
(**) Department of Gynecology and Obstetrics
Division of Gynecologic Oncology
Johann-Wolfgang Goethe University
Medical Center
Frankfurt/Main, Germany

that human tumors may induce suppressor cells or release factors that inhibit the function of infiltrating lymphocytes ([5, 7, 8]).

In the present study we have compared the growth of established tumor cell lines with the degree of mononuclear cell infiltration for further use as a therapeutic tool. We have studied the growth characteristics of TIL as well as their ability to proliferate in response to IL-2. In addition to that we have examined the growth characteristics, histochemical properties and antigen expression of the established ovarian adenocarcinoma cell lines.

MATERIALS AND METHODS

Patient population

The 12 patients in this study were diagnosed as having ovarian epithelial carcinoma, tumor diagnosis was proven histologically. The patients ranged in age from 32 to 68 years. Clinical data are listed in Table 1.

Cell preparation

Tumor samples were minced with scissors and treated for at least 45 minutes up to 3 hours with collagenase (10 mg/ml) and DNase (10 mg/ml) at 37 °C. After enzymatic digestion and filtration through a cell dissociation sieve, separation of tumor cells and lymphocytes was performed on two layers of Ficoll/Hypaque (75% and 100%) gradient as already described ([9, 10]). Afterwards the cells were counted and TIL were cultivated in microtiter plates (24 wells) whereas the tumor cells were cultivated in small cell culture flasks.

Culture conditions

TIL were cultured at a concentration of up to 5×10^5/ml in 24-well culture plates at 37 °C in a 5% CO_2 humidified atmosphere. As culture medium we used DMEM supplemented with 10% fetal salf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM) and nIL-2 (1000 U/ml). TIL were passaged every 5-7 days. The cultured tumor cells where treated similarly except that nIL-2 was omitted.

Analysis of lymphocyte surface markers

Surface markers of TIL were detected using a direct immunofluorescence assay. The monoclonal antibodies (mabs) used for flow cytometry were mouse anti-human either fluoresceine isothiocyanate- or phycoerithrine-conjugated (Becton-Dickinson, Heidelberg). They were directed against the following lymphocyte antigens: CD4, CD8, CD3, CD16, CD56 and CD45. After labelling with the fluorescence-conjugated mabs cells were stored at 4 °C in the dark until flow cytometry was performed. Lymphocytes were gated according to their smaller size and lesser granularity.

Expression of CA 125 antigen

For evaluation of CA 125 antigen in the culture supernatants a "microparticle enzyme immunoassay" (MEIA, Abbott) was performed. The test was kindly provided by M. Stegmüller, Division of Gynecologic Oncology.

Immunocytochemistry

For demonstrating the presence of CA 125 antigen on the surface of the tumor cells we used the "APAAP" technique as described earlier ([11]). The murine mab B 43.13 (Biomira, Canada), highly specific for CA 125, was used in the first step.

RESULTS

Yield of tumor cells

As the twelve human ovarian carcinomas of our patients differed in size and texture, the yield of prepared tumor cells showed wide varieties in number and growth. Table 1 shows the clinical data of the patients as well as the tumor histology. In comparison Table 2 shows the growth characteristics of the established cell lines. As 30% of the prepared tumor cell lines were of metastatic origin the question of interest was to investigate their growth characteristics versus those out of primary tumors. The results showed neither great differences concerning the time of culture periods nor in regard to the amount of grown tumor cells. The culture period of the cell lines ranged from 1 to 29 weeks with a bad, satisfactory, good or very good yield of grown tumor cells, respectively.

Yield of infiltrating lymphocytes

TIL could only be isolated from six out of twelve tumors (=50%). Their growth

Table 1. – *Source of tumor-infiltrating lymphocytes.*

Case	Age	Tumor histology	Survival after operation
K. D.	55	endometrioid carcinoma FIGO III	12 months
P. M.	62	solid carcinoma FIGO III	3 months
H. E.	68	Muellerian tumor FIGO III c	8 months
D. M.	32	papillary squamous cystadenocarcinoma FIGO III b	>20 months
E. E.	52	adeno-papillary ovarian carcinoma FIGO II c	>20 months
M. Re.	60	serous papillary adenocarcinoma FIGO III	>18 months
C. I.	57	cystadenocarcinoma FIGO I b	>18 months
M. Ri.	42	cystadenocarcinoma FIGO III c	>20 months
C. M.	44	papillary adenocarcinoma FIGO III c	19 months
R. G.	54	adeno-papillary carcinoma FIGO IV	18 months
S. I.	53	adeno-papillary carcinoma FIGO III c	>23 months
S. P. H.	56	papillary adenocarcinoma FIGO III	>15 months

rate (if any) was only poor (Table 3). Phenotyping by flow cytometry showed preferentially CD3+ cells (data not shown). The highest number of lymphocytes was 1×10^6 cells/ml, the lowest 2.3×10^5 cells/ml. The culture periods ranged from 2 weeks up to 9 weeks. During that time the cells did not react on stimulation with nIL-2 even though they were passaged regularly and supplemented weekly with fresh nIL-2.

CA 125 expression

Expression of the tumor marker CA 125 was evaluated in 8 out of 12 tumor cell lines tested. Values from 1.3 up to 38.2 U/ml could be found in the supernatants of the established cell lines. The expression of CA 125 was evaluated from 1 up to 6 months depending on growth

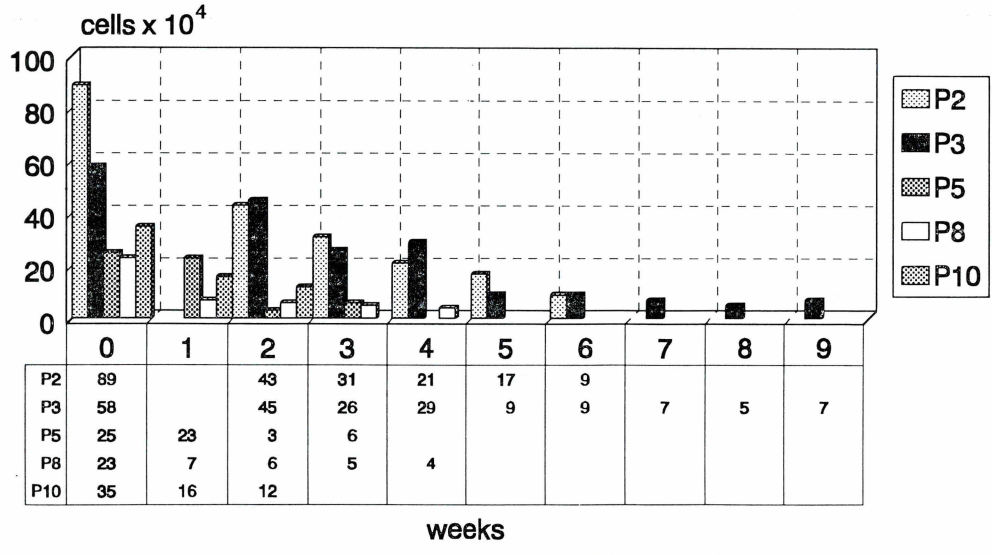
Table 2. – *Growth characteristics of isolated ovarian carcinoma cell lines.*

Sample	Growth*	Time**	Tipe of tumor
P1	0	1	primary
P2	3	29	metastasis
P3 a/b	0	10	primary
P3 c/d	3	22	metastasis
P4	2	20	metastasis
P5	1	8	primary
P6	0	7	metastasis
P7	2	15	primary
P8	3	21	primary
P9	1	7	primary
P10	0	5	primary
P11	3	9	primary
P12	3	3	primary

(*) growth: 3 very good, 2 good, 1 satisfactory, 0 bad.

(**) time: culture period in weeks.

Table 3. — *Growth characteristics of TIL from ovarian cancer patients in culture in the presence of nIL-2.*



	0	1	2	3	4	5	6	7	8	9
P2	89		43	31	21	17	9			
P3	58		45	26	29	9	9	7	5	7
P5	25	23	3	6						
P8	23	7	6	5	4					
P10	35	16	12							

and expansion rates of the tumor cells. The highest values could be found up to four weeks after isolation of the cells. In the following period the values slowly decreased. The expression of CA 125 served as a proof for growing ovarian carcinoma cells instead of fibroblasts.

Tumor cell growth versus growth of TIL

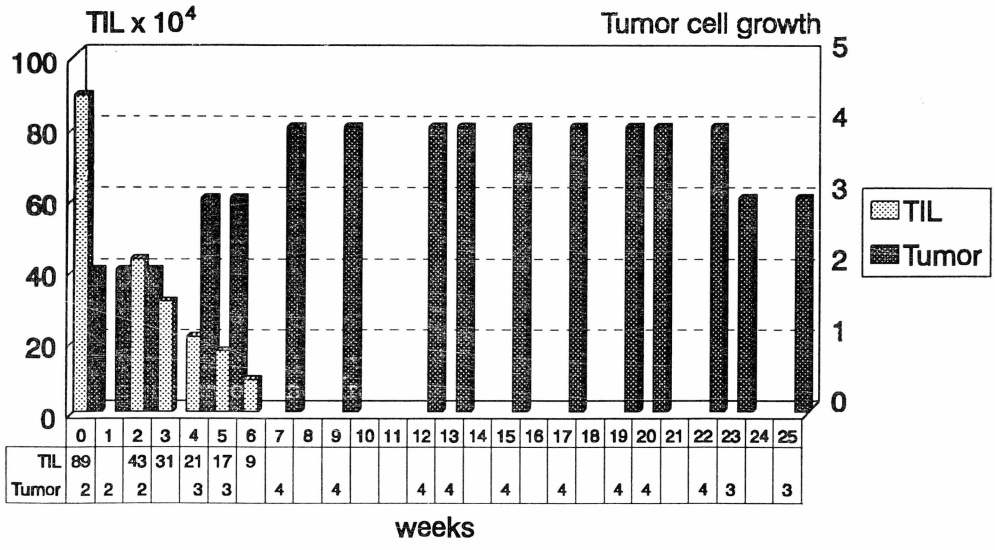
Tables 4 and 5 show two examples for the yield of tumor cells and of lymphocyte expansion. The tables show that the growth of the tumor cells was inversely correlated to the amount of TIL.

DISCUSSION

We have characterized 12 ovarian carcinoma cell lines as well as their lymphocytic infiltration. The immunohistochemical properties of the cell lines showed that most of them were a reasonable representation of the tumors in their natural state. Tumor-infiltrating lymphocytes, when activated with Interleukin-2 have been described to acquire an ability to destroy a variety of tumor cells and might be useful therapeutically ([12, 13]). Clinical trials were initiated to treat ovarian carcinoma patients with intraperitoneal tumor-infiltrating lymphocytes plus low-dose recombinant interleukin-2 ([14]). Lymphocytes infiltrating human ovarian tumors have already been characterized ([15]). Until now it has not been clearly established whether TIL are more effective than the circulating lymphocytes in adoptive therapy of different types of cancers. Only a small group (and primarily those with malignant melanoma) have been analyzed to date ([16]).

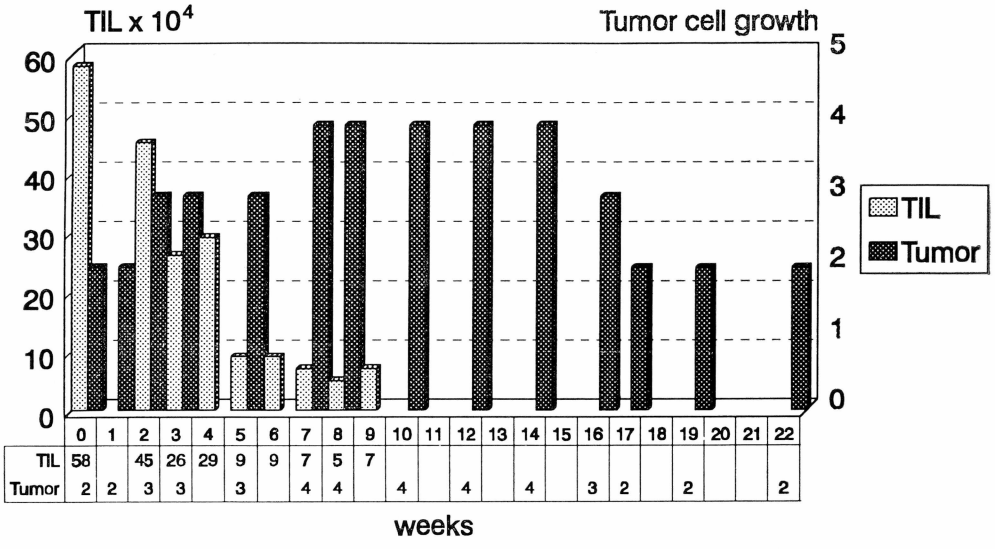
The aim of our study was to check the degree of infiltration in ovarian tumors and compare the yield of tumor cells and their expansion rate with the yield of TIL. Furthermore we wanted to find out whether TIL show any tumor-directed cytotoxic efficacy. Due to insufficient quantities and growth characteristics the

Table 4. — *Growth characteristics of tumor cells versus growth of TIL.*



	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
TIL	89		43	31	21	17	9																			
Tumor	2	2	2		3	3		4		4			4	4		4		4		4	4		4	3		3

Table 5. — *Growth characteristics of tumor cells versus growth of TIL.*



	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
TIL	58		45	26	29	9	9	7	5	7													
Tumor	2	2	3	3		3		4	4		4		4		4		3	2		2			2

cytotoxic activity of TIL could not be analyzed.

Based on our results a connection between tumor histology, amount of isolated TIL and growth characteristics of tumor cells cannot be verified.

CA 125 expression

Bast *et al.* ([17]) first reported elevated serum levels of CA 125 in 83% of patients with surgically demonstrable non-mucinous epithelial ovarian cancer. Due to its clinical value in pre-operative diagnosis and monitoring of ovarian cancer, CA 125 is the leading tumor marker in this disease ([18]).

The release of CA 125 into the culture medium was already studied in six human carcinoma lines, as well as the expression of CA 125 antigen on established ovarian cancer cell lines ([19]).

In conclusion the present investigation indicates that in terms of adoptive immunotherapy with human TIL it is not possible to establish conditions that may lead to outgrowth or generation of effectors with specific reactivity against autologous ovarian tumor cells.

Further work will have to be done to find treatment strategies concerning immunotherapy for ovarian carcinoma.

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Address reprints requests to:
B. DONNERSTAG
Zentrum der Biologischen Chemie
Universitätsklinik
Theodor-Stern-Kai 7
60590 Frankfurt/Main (Germany)