IMMUNOLOGIC CHARACTER OF TUMOR INFILTRATING LYMPHOCYTES IN OVARIAN CARCINOMA

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ABSTRACT

Objective: To study immunologic character of tumor-infiltrating lymphocytes (TIL) on post *in vitro* expansion in ovarian carcinoma, and evaluate the prospects by adopting TIL treatment of ovarian carcinoma at an advanced stage. Methods: Cellular phenotype changes in TIL were analyzed by flow cytometry. By means of molecular biology and immunologic methods, ability to secrete cytokines and anti-tumor activities of in TIL was studied. Results: Difference of cellular phenotypes in TIL was probably related to the type, feature and resource of the tumor. TIL obtained from phoroplast and parenchyma was dominant in $CD3^+CD4^+$. TIL obtained from tumor tissues, around microvessels and ascitic fluid was dominant in $CD3^+CD8^+$. Concentration of rIL-2 *in vitro* played a significant role in immunologic character of TIL. By means of rIL-2 expansion *in vitro*, TIL has apparently been improved in competence of secreting some cytokines, such as IL-2, TNF-α, IFN-γ, and anti-tumor activities.The activated TIL was more stimulated by further adding anti-CD3 or PHA (suitable concentration), which significantly increased its ability to secrete cytokines. Treatment with TIL+CTX or TIL+ rIL-2, could apparently improve phenotypes in peripheral blood of patients, with definitive effects. Conclusion: Immunologic activities of TIL *in vitro* are apparently improved by rIL2 expansion. Regression of tumor, by means of infusion TIL, is not largely attributed to direct cytotoxicity to tumor cells, but indirectly and partly augmenting cellular activities and abilities of immunomodulation in patients with ovarian carcinoma being dependent on secreting multiple cytokines.

Key words: Tumor-infiltrating lymphocytes, Cytokine, Ovarian carcinoma, Cellular phenotype

Tumor-infiltrating lymphocytes (TIL) was directly isolated from patient's tumor tissues. By rIL-2 activation and expansion *in vitro,* TIL was again imported into the same patient's body to treat tumor, with apparent effects of anti-tumor and comparative less side-effect, without killing other tumor cells and normal cells, all of which have made it a effective way of treating tumor at an advanced stage. In recent years, some researchers found that the effects of TIL treatment were not so far apparent, with comparatively apparent difference, which might be closely related to the fact that people didn't fully understand the character of TIL immunology, molecular biology et al.

In this article, we have studied biological activities, cellular phenotype, gene expression and secretion of some cytokines of TIL on post *in vitro* expansion in ovarian carcinoma, and have evaluated the effects of using TIL treatment of ovarian carcinoma at an advanced stage. The purpose of the research is to fully understand the function status of immunologic effect on cells of the patient with ovarian carcinoma, and to explore the mechanism of TIL anti-tumor cell *in vitro* in order to further develop the potential abilities of TIL anti-tumor, and to search for a more effective new way in the course of TIL adoptive immunotherapy of ovarian carcinoma at an advanced stage.

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MATERIALS AND METHODS

Patients

15 fresh specimens obtained from patients with ovarian carcinoma (mean age: 58±12 years) were provided by department of obstetrics and gynecology of Shanghai Rui Jing Hospital, Ren Jin Hospital and the Ninth People's Hospital. 11 of 15 cancers were primary epithelial. 2 of 15 cancers were metastatic recurrent. 2 of 15 cancers were serous.

Standard Raji, L929, CTLL2 cell strains

They were stored by our Department, cultured according to routine methods.

PCR Primer

Expansion of β -act, β -act$_1$, IL- 2, IL-2R(P55), IL2R(P75), TNF-α, IFN-γ, IL-4 and IL-6 primers (Shanghai SANGER ,LTD) were used in the research. The length of their expansion band was 660bp, 310bp, 458bp, 310bp, 371bp, 701bp, 468bp, 371bp, 408bp. The nucleotide sequences of their primers were described in references [2] in detail.

Isolation and Culfure of IIL Cells

Detailed procedure was described in references.[1,2]

Analysis of Phenotypes of TIL Cells

TIL cells with 95 percent of viable rate, during the stage of longarithm development, were obtained, disposed with CM and stayed overnight, in order to display their surface composition again. The cultured cells were collected once a week. The procedure of label was described in references[3] in detail. Then, 24-hour flow cytometry was used to detect the phenotypes of TIL cells.

Cytotoxicity Tests of TIL Cells

MTT method was performed. Detailed procedure was describe in references.[3] The intensity of radical ray was counted by β-counter. Specific killing activity was determined according to the formula as listed in below:

$$\text{Percentage of killing} = \frac{\text{(OD mean value of target cell control well} - \text{OD mean value of effect cell well} - \text{OD mean value of effect cell control well)}}{\text{OD mean value of target cells control well}} \times 100\%$$

Detection of Activity of IL-2 in Supernatant of TIL

Reaction of CTLL was performed by dependence on IL-2 cell strain. TIL cells cultured in 10 days were obtained. After the cells were washed twice in Hank's liquid, their concentration was regulated up to 1×10^6/ml, centrifuged at 500 × g for about 20 min. Supernatant was then harvested, and stored at -20 °C. The sample of supernatant was added into 96-well culture plate according to different diluted concentration, then detected and calculated. Detailed procedure was described in references.[4]

Detection of Activity of IFN-γ in Supernatant of TIL by Cell pathology inhi bitory method

Detailed procedure was described in references.[4]

Detection of Activity of TNF in Supernatant of TIL by L929 Cell Method

Detailed procedure was described in references.[5]

Detection of Expression of Gene of cytokines of TIL

Semi-quantitative RT-PCR was performed. Detailed procedure was described in references.[2,6]

Design of treatment by TIL

Design of TIL treatment was performed using chemistry sensitive anti-tumor drugs. Detailed procedure was described in references.[1,7,8]

Project of TIL Treatment

On the first day, highly sensitive anti-tumor drugs were used. On the second day, the side-effect of anti-tumor drugs was observed. From the third day to the fifteenth day, TIL cells ($>1\times10^9$/ml) mixed with rIL-2 ($2\sim5\times10^4$U/d) was intravenously dripped or partly injected into the patient's body. From the sixteenth day to twenty-fifth day, the patient was only injected rIL-2($2\sim5\times10^4$U/d). On the twenty-fifth day, highly

sensitive anti-tumor drugs were utilized again.

Statistical Analysis

The significance of observed differences was analyzed using χ^2 precise test.

RESULTS

Analysis of Cellular Phenotypes of TIL in Different Expansion Time

Expression of cellular phenotypes of 15 TIL samples was detected. On the tenth, twentieth, thirtieth day after expansion, percent of CD3, CD4, CD8, HLA-DR cells gradually expressing markers of T cell activation on TIL were found to increase, following extension of culture time, which explained that immunologic activities of TIL cells were gradually activated. The results were listed in Figure 1.

Analysis of Cellular Phenotypes of TIL (on the twentieth day after expansion by rIL-2 induction) Obtained from Different Sites

TIL, performing adoptive immunotherapy, largely consisted of mixed subgroups of lymphocytes, rather than a homogenous groups. Meanwhile, cellular phenotypes of TIL were analyzed. We found that TIL obtained from different sites was dominant in T cells, largely consisting of two subtypes of $CD3^+CD8^+$and $CD3^+CD4^+$, whose difference was probably related to the type, feature and resource of tumor. TIL obtained from fibroblast and parenchyma was dominant in $CD3^+CD4^+$. Whereas, TIL from tumor fibroblast, around microvessels and ascitic fluid, was dominant in $CD3^+CD8^+$. The results were listed in Table 1.

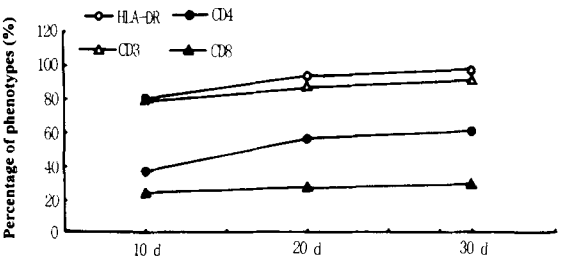

Fig. 1. Developmental changes of expression of markers of cellular phenotypes in TIL

Table 1. Analysis of cellular phenotypes of ovarian carcinoma TIL

Patient number	Resource	Percentage of positive cells (%)			
		CD3	CD4	CD8	HLA-DR
TIL was dominant in $CD4^+$					
2	Ovarian tumor	85.11	46.77	35.09	12.04
5	Metastatic lymph node from ovarian cancer	76.21	45.88	26.49	10.18
7	Ovarian tumor	83.24	57.30	19.70	85.90
9	Ovarian tumor	23.82	68.52	20.61	90.09
10	Ovarian tumor	42.78	33.05	24.40	93.60
12	Ovarian cancer ascitic fluid	83.24	57.30	19.70	85.90
13	Ovarian tumor	58.04	52.23	21.08	82.06
TIL was dominant in $CD8^+$					
1	Metastatic ovarian cancer from liver cancer	78.04	28.07	51.00	56.88
3	Ovarian cancer ascitic fluid	66.20	12.80	44.03	89.90
4	Ovarian cancer ascitic fluid	81.69	9.07	23.88	98.77
6	Ovarian cancer ascitic fluid	23.32	8.10	13.92	85.70
8	Ovarian cancer ascitic fluid	63.63	4.08	63.45	90.69
11	Ovarian cancer ascitic fluid	95.84	8.49	75.96	79.30
14	Ovarian tumor	77.00	26.60	53.00	86.60
15	Ovarian tumor	86.03	11.09	81.04	93.68

Killing activity of Ovarian Carcinoma TIL

7 samples of TIL dominant in $CD4^+$ and 8 samples of TIL dominant in $CD8^+$ had apparent killing activity against autogenous tumor cells, respectively, each of their activities was 98.89%, and 90.21% ($LU50/10^7$ cell, E:T=25:1). Meanwhile, all of them had definitely killing activity against K562 cells and heterogenous tumor cells. In different cultured time, the killing activity was different. From 10 days to 20 days, its killing activity of the cultured TIL was gradually increasing. Within the following 20 days, it increased to the peak. From 40 days to 56 days, comparable high killing activity of cultured TIL could still be kept. The results were listed in Table 2.

Table 2. Killing activity to ovarian cancer by TIL in different cultured days (%)

Cultured days	E : T				
	5:1	10:1	25:1	50:1	100:1
10 d	10± 1	18± 2	24± 3	30± 2	35± 4
20 d	28± 3	31± 2	57± 4	48± 4	71± 5
40 d	41± 4	27± 3	50± 4	52± 5	68± 5
56 d	38± 3	34± 3	47± 3	60± 5	69± 6

E: TIL cells T: Raji cells

Measure of Activity of IL-2, IFN-γ and TNF-α in Supernatant of Cultured TIL Cells Pro-and Post-expansion

Secretion of IL-2, IFN-γ was undetectable in 5 samples of supernatant before expansion. The mean unit of activity of secretion of IL-2, IFN-γ was apparently increased after expansion, whereas, the mean unit of activity of secretion of TNF-α post-expansion was apparently higher than pro-expansion. The results were listed in Table 3.

Table 3. Activity of some cytokines in supernatant of cultured TIL Cells pro-and post-expansion ($\bar{x}\pm s$, U/ml)

Patient	IL-2 (U/ml)		TNF-α (U/ml)		IFN-γ(U/ml)	
	Pro-expansion	post-expansion	Pro-expansion	post-expansion	Pro-expansion	post-expansion
1	0	45± 2	0	0	0	9± 2
2	0	13± 4	90± 3	120± 2	0	158± 3
3	0	50± 3	8± 1	20± 0	0	13±2
4	0	7± 1	5± 1	10± 3	0	36± 4
5	0	4± 2	40± 2	230± 2	0	45± 6
Average	0	24.0± 2	28.6± 1	76.0± 3	0	52.2± 4

Note: compared with pro- and post-expansion

Influence on Expression of mRNA of Some Cytokines of TIL Cultured with rIL-2 *in vitro*

TIL cells, by means of rIL-2 expansion *in vitro*, expressed β-actin, IL-2R (P55), IL-2R (P75), TNF-α, IFN-γ mRNA. However, 3 days after removed rIL-2, TIL didn't express mRNA of any cytokines. By using high concentration of rIL-2 (1000~2000 U/ml) expression *in vitro*, β-actin, IL-2R(P55), IL-2R(P75), TNF-α, IFN-γ mRNA were expressed by TRIL cells. In contrast, TIL cells only expressed β-actin, IL-2R(P75) mRNA by using low concentration of rIL-2 (100 U/ml) expansion. The results were listed in Table 4 and Figure 2.

Table 4. Influence on expression of mRNA of some cytokines of TIL with rIL-2 in vitro

	IL-2R		TNF-α	IFN-γ	Il-2
	P55	p75			
rIL-2 stimulation	0.975	1.185	1.213	0.374	0
rIL-2 post-remove*	0	0	0	0	0
rIL-2 (1000U/ml)	0.925	0.951	0.931	0.892	0
rIL-2 (100U/ml)**	0	0.643	0	0	0

Note: rIL-2 pro-and post-remove* $P<0.01$ rIL-2 low and high concentration** $P<0.01$

Influence on Expression of mRNA of Some Cytokines of TIL Cultured with rIL-2+anti-CD3 or rIL-2+PHA

Compared expansion of TIL using only rIL-2 with using rIL-2+anti-CD3 or rIL-2+PHA, we found that the latter could apparently enhance expression of IL-2R, TNF-α, IFN-γ mRNA, and produce IL-6, IL-2, IL-4. The results were listed in Figure 3.

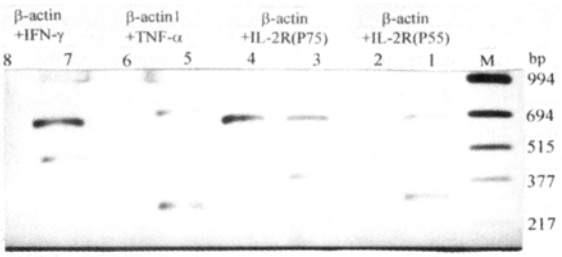

Fig. 2. Influence on expression of mRNA of some cytokines of TIL cultured with different concentration of rIL-2

Lane 1, 3, 5, 7: Products of high concentration of rIL-2 (1000 U/ml)

Lane 2 ,4, 6, 8: Products of low concentration of rIL-2 (100 U/ml)

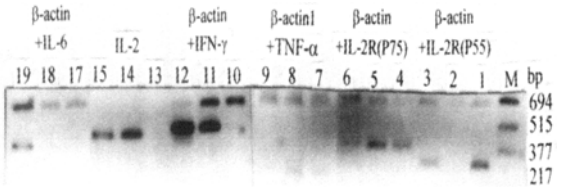

Fig. 3. Influence on expression of mRNA of some cytokines of TIL cultured with rIL-2+ anti-CD3.

Lane 1, 4, 7, 10, 13, 17: rIL-2

Lane 2, 5, 8, 11, 14, 18: PBMC control

Lane 3, 6, 9, 12, 15, 19: rIL-2+anti-CD3

Analysis of Phenotypes of Peripheral Blood T-cells Pro- and Post-clinic Treatment with TIL

With application to treatment projects of this research, phenotypes of peripheral blood T-cells pro- and post-treatment were detected by using flow cytometry. We found that the rate of $CD3^+$, $CD4^+$ $CD8^+$, NK and LAK cells contained in the subtype of patient's peripheral blood T-cells, acting as T-cells marker of activation, were apparently increased after treatment, and immunologic functions of T-cells were further enhanced. The results were listed in Table 5, figure 4.

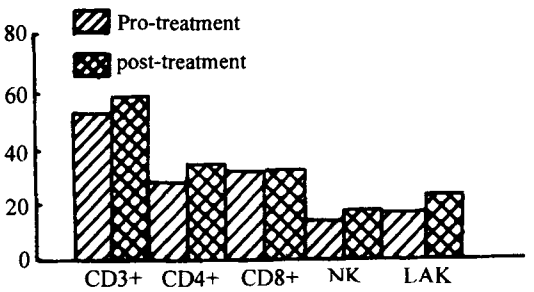

Fig. 4. Changes of phenotypes of peripheral blood T-cells of patients with ovarian cancer pro- and post-treatment with TIL

Table 5. Analysis of phenotypes of peripheral blood T-cells pro- and post-clinic treatment with TIL($\bar{x}\pm s$)

Group	Patient numbers	$CD3^+$	$CD4^+$	$CD8^+$	NK	LAK
Pro-treatment	8	52.5± 2.1	27.5± 1.9	30.8± 2.5	11.5± 3.5	16.12± 2
Post-treatment	8	60.8± 2.5	33.1± 3.5	31.5± 1.5	17.3± 3.2	19.65± 3

Note: compared with pro-and post treatment $P<0.05$.

DISCUSSION

TIL, following behind LAK cells, was the second generation cell having anti-tumor immunologic effect in adoptive therapy. In recent years, TIL has been widely utilized in clinic to treat patients with carcinoma at an advanced stage, with definitely recoverable effects. However, with the depth of research, people have found that there were many apparent differences in estimating TIL biotherapy effects, producing many troublesome problems. Therefore, it was fully meaningful for us to understand deeply the character of TIL immunity, molecular biology et al.[9]

Before ovarian TIL freshly excised from tumor issue expanded, its immunologic activity was quite low, which was probably related to the fact that some inhibitory cytokines, such as, IL-10, TGF-β et al,[10,11] were expressed and secreted by ovarian cancer cells. On the tenth, twentieth, thirtieth day after expansion, HLA-DR, as marker of activated TIL, was further expressed. TIL cells largely consisted of many subtypes of lymphocytes, rather than a homogenous groups. Different sites obtained from the tumor showed that the character of tumor had great influence on major subtypes of TIL groups, in some degree. So, it also influenced different effects of various treatment.

TIL, after expansion, had very high killing ability against autogenous tumor cells and also comparably powerful functional specificity. Generally speaking, after TIL was expanded on the second, third week, its killing activity increased to the peak. This phase was the best time for adoptive immunotherapy. By measure of activity of cytokines in supernatant of

cultured TIL, we found that TIL, after expanded, could secrete high activity of TNF-α、IFN-γ、IL-2 et al.

We have also further confirmed that TIL could express those genes of cytokines by means of RT-PCR. However, expression of TNF-α mRNA could be kept for a long time. Meanwhile, expression of IL-2 mRNA was transient, and expression of IFN-γ mRNA was sometimes missing. What needed to point out that IL-2 and IFN-γ were both important immunologic regulation factor during delayed hypersensitivity, whereas, TNF-α was a cytokine with apparent ability of anti-tumor effects. Therefore, we can draw a conclusion that regression of tumor, by means of infusion TIL, is not largely attributed to direct cytotoxicity to tumor cells, but indirectly and partly augmenting cellular activities and abilities of immunomodulation in patients with ovarian carcinoma, being dependent on secreting multiple cytokines.

The activated TIL, was removed from rIL-2, after 3 or 5 days, none of any cytokine was found to secrete. It was demonstrated that activation of TIL in human body was greatly dependent on rIL-2. For this reason, in studying their relationships, we used different concentrations of rIL-2, and found that high concentration of rIL-2 (1000~2000 U/ml) could significantly expand TIL. But TIL decreased its killing specificity for autogenous tumor, with non-antigen specificity and non-MHC restrictive cytotoxity. After TIL was expanded by low concentration of rIL-2 (100U/ml), we found that the growth of TIL was kept stagnant and expression of cytokines was inactive. The activated TIL was more stimulated by further adding anti-CD3, or PHA (suitable concentration), which increased its ability to secrete IL-2R、TNF-α、IFN-γ. Furthermore, TIL could porduce IL-6、IL-2、IL-4. It suggested that adding anti-CD3 or PHA, in some degree, could overcome insufficiency of TIL effective reproduction and specific killing ability in culture of low concentration of rIL-2.

Based on above mentioned researches, we have also developed that some ovarian carcinoma at an advanced stage were additionally treated by using TIL adoptive immunotherapy, and found that the immunologic functions of patients. Peripheral blood T-cells, after the whole course (about 3 months), could be apparently improved, which demonstrated that TIL treatment was definitely effective. With regard to the assessment of TIL treatment effects, we are still in further studying.

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