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Semiquantitative analysis of Th1 and Th2 cytokine expression in CD3$^+$, CD4$^+$, and CD8$^+$ renal-cell-carcinoma-infiltrating lymphocytes

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Abstract The mRNA expression of Th1 and Th2 cytokines was compared in freshly isolated CD3$^+$ tumor-infiltrating lymphocytes (CD3$^+$ TIL) and in autologous CD3$^+$ peripheral blood lymphocytes (CD3$^+$ PBL) obtained simultaneously from 20 patients with renal cell carcinomas (RCC). In addition cytokine expression was compared in CD4$^+$ TIL and CD8$^+$ TIL from another group of 20 patients with RCC. TIL were isolated from mechanically disaggregated tumor material and PBL from peripheral blood by gradient centrifugation and subsequent selection with anti-CD3, anti-CD4 or anti-CD8 magnetic beads. In these pure lymphocyte preparations the constitutive expression of interleukin-1 (IL-1), IL-2, IL-10, interferon γ (IFNγ), and tumor necrosis factor α (TNFα) was determined by using a polymerase-chain-reaction-assisted mRNA amplification assay. In the CD3$^+$ TIL, levels of mRNA for IFNγ, IL-10, IL-1 and TNFα were significantly higher than in the autologous CD3$^+$ PBL whereas IL-2 expression was rather low and did not differ in the two populations. Comparison of cytokine mRNA expression in CD4$^+$ TIL and simultaneously obtained CD8$^+$ TIL revealed a significantly higher expression of IFNγ in the CD8$^+$ cells. These data reflect an in vivo activation of RCC-infiltrating lymphocytes at the mRNA level with respect to the Th1 as well as the Th2 immune response. Th1 activation seems to be most evident in the CD8$^+$ TIL.

Key words Renal cell carcinoma · TIL · Cytokine expression

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Introduction

Renal cell carcinomas (RCC) are generally infiltrated by lymphocytes (TIL) and this infiltrate has been considered to be part of the host's immune response against the tumor [13, 17]. However, despite an apparent local accumulation of lymphocytes, including cells with the potential to respond to autologous tumor cells [6, 8, 12, 22] progressive tumor growth occurs. Recent findings of alterations in surface receptors required for T cell proliferation [11, 15, 24], as well as a poor proliferative response [1], the failure to grow TIL from some tumor samples [27] and the variable results with TIL therapy in clinical trials [7, 20] have led to the suggestion that TIL in RCC may be compromised in their antitumor efficacy. Local cytokine production is thought to play a central regulatory role in the activation of tumor-associated inflammatory cells and may have an impact on the development of an effective antitumor response. However, little is yet known about the immunological activation of infiltrating lymphocytes in the tumor environment. Since expression of various cytokines has been shown to be induced during T cell activation, the aim of the present study was to characterize freshly isolated CD3$^+$, CD4$^+$ and CD8$^+$ TIL populations with respect to their expression of mRNA for Th1-type and Th2-type cytokines.

Materials and methods

Tumor and blood samples

Tumor material and heparinized peripheral blood samples were obtained from 40 patients with primary renal cell carcinomas undergoing therapeutic surgery. Blood was taken at the onset of surgery. None of these patients had received preoperative antitumor therapy. Procedures to separate blood and tumor samples were started within 30 min after the operation.

In the first group of 20 RCC patients, CD3$^+$ TIL and simultaneously obtained autologous CD3$^+$ peripheral blood lympho-

cytes (PBL) were isolated and compared for their expression of cytokine mRNA.

In a second group of 20 RCC patients, $CD4^+$ TIL were compared with autologous $CD8^+$ TIL.

Isolation of $CD3^+$, $CD4^+$ and $CD8^+$ TIL and PBL

After removal of necrotic areas and fat, the tumor specimens were washed in phosphate-buffered saline (PBS), minced to small pieces, washed again in PBS and then gently homogenized in a "loose-fitting" hand homogenizer. In order to avoid all aggressive methods and long preparation times no enzymatic digestion was applied. The resulting single-cell suspensions containing TIL and tumor cells were overlayered onto 30% Percoll (Pharmacia, Freiburg, Germany) and centrifuged at 400 *g* for 20 min at room temperature. The sediment was diluted in RPMI medium, overlaid onto Ficoll-Paque (Pharmacia) and centrifuged at 400 *g*. From the cells of the interphase, $CD3^+$, $CD4^+$ or $CD8^+$ lymphocyte populations were selected, using magnetic beads coated in monoclonal antibodies to these determinants (Miltenyi, Bergisch Gladbach, Germany).

PBL were isolated from heparinized blood by Ficoll density centrifugation. Mononuclear cells were collected from the interphase and washed twice. $CD3^+$, $CD4^+$ or $CD8^+$ cell fractions were selected with magnetic beads. Cells were counted and 10^5 cells were lysed in 350 µl solution consisting of 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sodium *N*-dodecylsarcosinate and 100 mM 2-mercaptoethanol.

Quantification of cytokine mRNA expression by reverse transcription/polymerase chain reaction (RT-PCR)

Total RNA was isolated with silica-gel-based membranes (Rneasy; Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA synthesis was performed at 42 °C for 60 min in a final volume of 50 µl, which contained 25 µl denatured RNA, 10 µl 5× buffer (Promega, Heidelberg, Germany), 5 µl 10 mM dNTP (dATP, dCTP, dGTP, dTTP, Promega), 1.5 µl RNAsin (40 units/µl, Promega), 2.5 µl 150 pM random hexamer primers, and 2.5 µl avian myeloblastosis virus reverse transcriptase (10 units/µl, Promega).

Samples were subsequently diluted in 1:3.16 steps and 2-µl aliquots were combined with the PCR mixture, containing 11 µl water, 2 µl 10× buffer (Promega), 2 µl 10 mM dNTP, 1 µl 25 pM sense and antisense primer, and 1 µl (1 U) Taq polymerase (Promega). For all cytokines and β-actin, internal standards had been constructed that were added to the PCR reactions at a constant low amount (unpublished data).

The reaction mixture was amplified with a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) for 33 cycles. To exclude the possibility that contaminating genomic DNA might give interfering misleading signals, each lymphocyte RNA preparation was also tested with each primer set directly without reverse transcription.

Prior to testing for the presence of cytokine-related transcripts, a PCR using a primer set to amplify β-actin was run at several dilutions in order to prove the integrity of the extracted RNA and to standardize the template. Because it is known that a number of processed β-actin pseudogenes exists [19, 21], a primer set 5′-ACGGCTGCTTCCAGCTCCTC-3′ and 5′-TTGTTTTCTGCGCAAGTTAG-3′ (62 °C) was chosen that had been proven not to amplify pseudogenes in control experiments without reverse transcription.

To exclude contamination with tumor cells, each TIL RNA preparation was tested with a primer set for human carbonic anhydrase XII, a gene that is expressed in renal cancer cells [25]. With the primer set 5′-CCGCCGAGCTGCACATTGTC-3′ and 5′-GGGCCAGTGAGAGGATGATG-3′ we found specific signals in all RCC tumor samples tested, but not in lymphocytes. Experiments with mixtures of cells showed that, with 33 cycles, a tumor cell contamination in lymphocytes of less than 1% could be easily detected. Only TIL samples without detectable tumor cell contamination were used for further analysis. (unpublished data).

The following oligonucleotide 5′ and 3′ primer sequences for the cytokines with their annealing temperatures (in brackets) were used: IL-1, 5′-CCTCTAAAACATCCAAGCTTACCA-3′ and 5′-CGTCTAGAGTTCTTAGTGCCGTGAGTTTC-3′ (58 °C); IL-2, 5′-CAGTGTTGAGATGATGCTTTGA-3′ and 5′-ATGTACAG GATGCAACTCCTGTCT-3′ (64 °C); IL-10, 5′-ATGGGGGTT GAGGTATCAGAGG-3′ and 5′-ATGCCCCAAGCTGAGAACC AAG-3′ (64 °C); interferon γ (IFNγ), 5′-ACCGAATAATTAGT CAGCTT-3′ and 5′-AGTTATATCTTGGCTTTTCA-3′ (54 °C); tumor necrosis factor α (TNFα), 5′-GGGGTACCTGGAAAG-GACACCATGA-3′ and 5′-GCTCTAGACCTTGGTCTGGTAG GA-3′ (58 °C).

PCR products were separated on 6% polyacrylamide gels and detected with silver staining (Pharmacia). Gels were scanned for documentation. Gel bands were measured densitometrically and pixel intensities of those bands that were in the linear range and most comparable for TIL and PBL were evaluated and multiplied by the dilution factor. Identification of the amplification products was done with restriction endonuclease analysis, sequence analysis and size determination. Sizes of the amplified fragments were as follows: IL-1, 530 bp; IL-2, 457 bp; IL-10, 410 bp; IFNγ, 357 bp; TNFα, 527 bp.

Statistical methods

The relations between mRNA expression (pixel intensity of the PCR bands multiplied by the dilution) in the corresponding $CD3^+$ TIL and $CD3^+$ PBL, as well as in the autologous $CD4^+$ TIL/$CD8^+$ TIL pairs, were statistically evaluated by the paired Wilcoxon test.

Results

Expression of mRNA for cytokines in $CD3^+$ TIL compared to autologous $CD3^+$ PBL

From 5 g tumor material the recovery of $CD3^+$ TIL by specific enrichment with monoclonal-antibody-coated magnetic beads was between 10^5 and 10^6 cells, the recovery of $CD4^+$ and $CD8^+$ TIL was between 5×10^4 and 8×10^5 cells. Therefore it was not possible to isolate both $CD3^+$ and $CD4^+/CD8^+$ TIL from the same patient.

Prior to the analysis of cytokine-gene expression by PCR-assisted mRNA amplification in $CD3^+$ TIL and $CD3^+$ PBL, the samples were standardized for the mRNA levels of β-actin, so that equal amounts of template were used in the corresponding autologous TIL/PBL pairs. In addition, in each TIL RNA sample a PCR for human carbonic anhydrase XII was run to exclude contamination with tumor cells.

PCR experiments to semiquantify the expression of the different cytokines were run in multiple dilutions. Corresponding PCR bands of $CD3^+$ TIL and autologous $CD3^+$ PBL, which always derived from the same experiment, were quantified densitometrically and compared.

In the $CD3^+$ TIL from 20 renal cell carcinomas, a significantly higher mRNA expression of IFNγ ($P = 0.0000$), IL-10 ($P = 0.0153$), IL-1 ($P = 0.0268$) and TNFα ($P = 0.0484$) was found than in the auto-

logous $CD3^+$ PBL, whereas the mRNA levels of IL-2 were rather low throughout and not significantly different in $CD3^+$ TIL and $CD3^+$ PBL. Data are summarized in Table 1.

In order to prove that the somewhat different purification of the TIL (mechanical disaggregation, Percoll density centrifugation) does not induce a measurable cytokine expression in lymphocytes, in some experiments PBL were treated in the same way as TIL. In these experiments it could be shown that neither the mechanical treatment in the homogenizer nor Percoll density centrifugation induced cytokine expression (data not shown).

In addition, by comparison of $CD3^+$ PBL obtained by depletion or by positive selection, it could be demonstrated that the short incubation time with CD3 magnetic beads does not affect cytokine mRNA expression in these cells.

mRNA expression of cytokines in $CD4^+$ TIL compared to autologous $CD8^+$ TIL

In a second group of another 20 RCC patients, cytokine expression was compared in $CD4^+$ TIL and autologous $CD8^+$ TIL. With a PCR for human carbonic anhydrase XII, any possibility of contamination with tumor cells in the samples could be excluded. After standardization of the mRNA levels of each $CD4^+/CD8^+$ TIL pair for β-actin, expression of the mRNA for IL-10, IFNγ, and TNFα was quantified.

In the $CD8^+$ TIL the mRNA levels for IFNγ were significantly higher than in the autologous $CD4^+$ TIL ($P = 0.0009$), whereas the mRNA levels for the other cytokines were not significantly different in the two TIL populations. Data are summarized in Table 2.

Discussion

The ability to study functional parameters of TIL reliably is largely dependent on their successful, i.e. gentle and pure, separation from tumor biopsies. In former studies, mostly TIL preparations separated from tumor material by gradient centrifugation were investigated and these were reported to contain 10%–95% lymphocytes [4, 28] and 6%–75% tumor cells [4]. In contrast to these reports, the positive selection method with magnetic beads used in the present study resulted in pure $CD3^+$, $CD4^+$, and $CD8^+$ TIL populations, which were free from tumor cells. In these lymphocyte preparations we could show a higher expression of IL-10, IL-1, IFNγ and TNFα in the $CD3^+$ TIL than in the $CD3^+$ PBL.

In the literature, there are only few comparable studies of cytokine mRNA levels in TIL from RCC. Our finding of high IL-10 mRNA levels in TIL is in accordance with those of Wang et al., who detected IL-10 mRNA in freshly isolated lymphocyte-enriched preparations from 4/5 RCC tumor specimens [26], and Maeurer et al., who found that uncultured TIL from seven RCC patients typically exhibited IL-10 and IL-4 mRNA [16]. Other authors detected high IL-10 mRNA levels in RCC tumor samples, but not in RCC cell lines, peripheral blood mononuclear cells and non-tumorous kidney tissues [18]. High levels of TNFα mRNA transcripts have

Table 1 Relative mRNA levels of cytokines in freshly isolated $CD3^+$ tumor-infiltrating lymphocytes (*TIL*) and autologous $CD3^+$ peripheral blood lymphocytes (*PBL*) from 20 patients with renal cell carcinoma. cDNA of each TIL/PBL pair was standardized for β-actin mRNA levels. Gel bands were measured densitometrically and pixel intensities were multiplied by the dilution. Mean values are given and, for each combination, the highest number is in bold type. *IFN* interferon, *IL* interleukin, *TNF* tumor necrosis factor

Patient no.	INFγ		IL-10		TNFα		IL-1		IL-2	
	$CD3^+$ TIL	$CD3^+$ PBL	$CD3^+$ TIL	$CD3^+$ PBL	$CD3^+$ TIL	$CD3^+$ PBL	$CD3^+$ TIL	$CD3^+$ PBL	$CD3^+$ TIL	$CD3^+$ PBL
3	**20 000**	14 000	**2000**	1100	**37 000**	36 000	**7500**	4700	**1200**	560
5	**62 000**	13 000	**57 000**	4000	0	**4700**	**17 000**	15 000	0	**4500**
8	500	**640**	5700	**52 000**	500	**950**	**480**	430	**470**	320
9	**59 000**	3200	**70 000**	18 000	**24 000**	6900	2300	**2500**	**25 000**	43
10	**5400**	**9100**	1100	**21 000**	1800	**4300**	88	**9700**	520	**1300**
11	**99 000**	2800	**23 000**	2700	**23 000**	2600	**7400**	1700	**18 000**	0
12	**6400**	610	**2100**	310	**7900**	2700	**6000**	1700	0	**730**
14	**4700**	510	**5000**	3200	**3500**	1300	**690**	240	0	0
15	**4700**	0	**800**	0	660	**1300**	0	0	0	0
16	**4800**	2000	650	**1500**	8100	**9300**	1200	**1500**	**1600**	860
17	**11 000**	1400	1000	**1100**	**990**	730	1500	**1800**	**1900**	0
18	**48 000**	4000	**21 000**	5100	**67 000**	3000	**30 000**	4500	**19 000**	11 000
19	**18 000**	2000	**4600**	3600	**19 000**	1200	**11 000**	2100	**450**	0
23	**320**	150	**6600**	2700	**670**	610	**180**	0	100	**4100**
24	**7400**	620	**38 000**	3600	**4700**	320	**7500**	770	**640**	0
31	**5500**	2000	**28 000**	5300	29 000	**30 000**	**9400**	7200	**130**	0
41	**1900**	200	**52 000**	14 000	**2200**	980	**4100**	600	**460**	0
42	**17 000**	4000	**15 000**	4100	**74 000**	12 000	**18 000**	17 000	0	0
43	**13 000**	4300	**52 000**	17 000	**15 000**	14 000	34 000	**38 000**	**2200**	0
44	**3600**	280	**1100**	250	**12 000**	2700	0	0	0	**470**

Table 2 Relative mRNA levels of cytokines in freshly isolated $CD4^+$ TIL and $CD8^+$ TIL of 20 patients with renal cell carcinomas. cDNA of each $CD4^+$ TIL/$CD8^+$ TIL pair was standardized for β-actin mRNA levels. Gel bands were measured densitometrically and pixel intensities were multiplied by the dilution. Mean values are given in the table and, for each combination, the highest number is in bold type

Patient no.	IFNγ		TNFα		IL-10	
	$CD4^+$ TIL	$CD8^+$ TIL	$CD4^+$ TIL	$CD8^+$ TIL	$CD4^+$ TIL	$CD8^+$ TIL
2	12 000	**62 000**	**42 000**	9500	3100	**27 000**
3	**51 000**	29 000	15 000	**16 000**	**8900**	2800
5	9200	**26 000**	9900	**10 000**	**23 000**	17 000
6	**40 000**	13 000	**1600**	800	**22 000**	630
7	12 000	**13 000**	47 000	**56 000**	**18 000**	11 000
8	0	**1300**	**4800**	1600	0	0
9	11 000	**41 000**	**18 000**	10 000	16 000	**20 000**
12	590	**3000**	**16 000**	8500	4000	**5300**
13	2700	**4100**	2100	**2200**	**9300**	970
14	420	**560**	2300	**5600**	1800	**3300**
15	2400	**4900**	**38 000**	18 000	110 000	**240 000**
16	2700	**19 000**	7700	**16 000**	5700	**13 000**
17	810	**3800**	3400	**7400**	3300	**25 000**
18	240	**3300**	27 000	**100 000**	14 000	**50 000**
19	11 000	**19 000**	42 000	**79 000**	17 000	**29 000**
20	2000	**3300**	8000	**12 000**	2100	**9100**
21	**3300**	3200	**8600**	8200	**6100**	4400
22	3200	**7500**	9000	**9100**	12 000	**13 000**
23	8300	**32 000**	14 000	**34 000**	40 000	**650 000**
24	25 000	**41 000**	30 000	**47 000**	**47 000**	28 000

also been described in RCC TIL [5]. Our finding of a high IFNγ expression in TIL seems to be in contrast to those of two other publications, which emphasized a decrease of IFNγ mRNA in TIL from renal cell carcinomas [10, 23]. However, one of these studies used long-term cultured and stimulated TIL while, in the other, tumor biopsies were investigated, and therefore results are not comparable. Using freshly isolated TIL rather than cultured cells may better represent the in vivo situation.

To our knowledge, the present study is the first to investigate mRNA levels of Th1 and Th2 cytokines systematically in freshly isolated, pure $CD3^+$, $CD4^+$, and $CD8^+$ TIL from a statistically relevant number of renal cell carcinomas. Our data showing a higher cytokine gene expression at the mRNA level in $CD3^+$ TIL than in autologous $CD3^+$ PBL may reflect an activation of the immune cells in the tumor environment.

From the concomitantly high levels of mRNA for IL-10 and IFNγ in $CD3^+$ TIL, found in our study, it may be concluded that Th1 as well as Th2 lymphocytes are activated, which is in accordance with a recently published paper also showing high levels of IL-10 and IFNγ in TIL from non-small-cell lung cancer biopsies [3]. In contrast, when $CD4^+$ TIL were compared with autologous $CD8^+$ TIL, only IFNγ was significantly more highly expressed in the $CD8^+$ TIL, which may indicate a preferential Th1 activation of these cells.

However, cytokine mRNA expression may not necessarily indicate that these cytokines are also secreted as proteins, which is suggested to be necessary for an antitumor response. Therefore it is currently unclear whether the $CD8^+$ TIL represent a cytolytically active population or, whether in spite of activation, they may be anergic because of posttranscriptional mechanisms or inhibitory factors produced by the neoplastic cells.

Indeed, tumor cell growth is likely to be influenced not only by the ability of cytolytic T cells to recognize and respond to the tumor cells but also by the cytokine environment at the tumor site. Whereas isolated TIL have been shown to have a normal capacity to produce cytokines in vitro upon stimulation with anti-CD3 or mitogens [2, 9], cytotoxicity data with freshly isolated $CD8^+$ TIL suggest that these cells may not fulfill an effector function in vivo [14]. Understanding the immunoactivating and suppressive effects in the tumor environment may help the development of new immunotherapeutical strategies. Long-term studies correlating cytokine expression in TIL with the clinical outcome may help us to understand the effectiveness of these cells.

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