Favorable prognosis of renal cell carcinoma with increased expression of chemokines associated with a Th1-type immune response

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The potential role of chemokines in clinical tumors remains poorly understood. Recent investigations have shown the differential expression of chemokine receptors on lymphocytes mediating Th1- and Th2-type immune responses. We examined Th1- and Th2-associated cytokines and chemokines, as well as the expression of their receptors in tumor-infiltrating lymphocytes in renal cell carcinoma (RCC). Sixty-seven patients with sporadic RCC were analyzed for the expression of Th1- and Th2-associated genes using real-time polymerase chain reaction. Tumor infiltration by CXC chemokine receptor 3 (CXCR3)-positive and CC chemokine receptor 5 (CCR5)-positive cells was detected by immunohistochemistry and by flow cytometry. The expression of Th1-associated genes was significantly increased in tumors compared to normal kidney tissues. The expression of interferon-γ correlated positively with that of Th1 chemokines. Tumors expressing higher Th1 chemokines did not recur after curative surgery. Multivariate analysis showed that increased monokine induced by interferon (IFN)-γ (MIG) expression was an independent favorable prognostic factor. Immunohistochemistry showed that the degree of CXCR3-positive cell infiltration significantly correlated with IFN-γ inducible protein 10, MIG and IFN-γ-inducible T cell a chemoattractant expression (I-TAC). Flow cytometric analysis showed increased expression of CXCR3 and CCR5 in tumor-infiltrating T lymphocytes compared to that in peripheral blood T cells. These results suggest that upregulation of the Th1-type immune response in RCC tumors with a favorable prognosis may be mediated by Th1-associated chemokines. Integrity of the Th1-type immune response seems to be required for tumor regression, suggesting that detection and correction of a defect in the Th1-type response cascade would thus be one of the main targets for tailor-made immunotherapy and gene therapy in RCC. (*Cancer Sci* 2006; 97: 780–786)

Chemokines are a superfamily of small, proinflammatory molecules that regulate leukocyte trafficking in the body.[1] Chemokines can be divided into four families according to structural homology: the CXC, the CC, the C and the CX_3C families.[2–5] In addition to chemoattraction, recent investigations have demonstrated the possible role of chemokines in tumor growth and metastasis, and in the host-tumor response.[6] Various chemokines have been demonstrated to be antitumorigenic in some neoplasms,[7–9] whereas some chemokines appear to have tumorigenic and metastatic potential.[10,11] However, the expression and potential role of chemokines in human neoplasms *in vivo* remains to be characterized.

Renal cell carcinoma (RCC) is known as a hypervascular neoplasm in which the host antitumor response appears to influence tumor growth by inducing cellular infiltration.[12] We hypothesized that chemokines may play an important role in regulating tumor growth in RCC. We examined chemokine gene expression in RCC tumors, and recently reported the increased expression of some chemokine genes in tumors with a favorable prognosis, including interferon (IFN)-γ inducible protein 10 (IP-10), monokine induced by IFN-γ (MIG), macrophage inflammatory protein (MIP)-1β and regulated upon activation normal T expressed and secreted (RANTES).[13] This 2004 study suggested that the antitumorigenic activities of these chemokines may result from the recruitment of tumor infiltrating lymphocytes to amplify the antitumor immune responses.[13]

It is known that immune responses can be subdivided according to the kind of cytokines produced from $CD4^+$ T helper cells.[14–16] T helper cells type 1 (Th1) secrete IFN-γ and mediate cellular immunity, whereas Th2 producing interleukin (IL)-4, IL-5 induce the humoral immune responses. Recent investigations have shown the differential expression of chemokine receptors in Th1 versus Th2, suggesting that the expression pattern of chemokines may determine the polarization of the immune response to Th1 or Th2.[17–19] Th1 preferentially express the CXC chemokine receptor 3 (CXCR3), the receptor for IP-10, MIG and IFN-γ-inducible T cell a chemoattractant (I-TAC), the receptor for CC chemokine 5 (CCR5), a receptor for MIP-1β, and RANTES. In contrast, Th2 mainly express CCR3, a receptor for eotaxin, and CCR4, a receptor for macrophage-derived chemokine (MDC). Thus, in the present study, we designated IP-10, I-TAC, MIG, MIP-1β and RANTES as Th1-associated chemokines, and MDC and eotaxin as Th2-associated chemokines.

On the basis of our previous study, we speculated that the recruitment of tumor-infiltrating lymphocytes may be one of the mechanisms for the antitumorigenic effects of chemokines

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in RCC. These studies raised questions regarding what type of immune response is amplified in a tumor with a favorable prognosis. In the present study, we compared the expression of chemokines preferentially recruiting Th1 and Th2, and the cytokines involved. We also analyzed the expression of chemokine receptors in tumor-infiltrating cells.

Materials and Methods

Patients and sample collection

Tissue samples were collected from 67 patients with RCC who underwent surgery in our department (Urology, Tokyo Women's Medical University). The samples were taken from the tumor, along with normal kidney tissue of the nephrectomy specimens, and stored in liquid nitrogen until preparation for analysis. Tumors were staged according to the 1997 Union Internacional Contra la Cancrum, and the American Joint Committee on Cancer TNM classification for kidney cancer.[20] All patients were evaluated for postoperative recurrence by blood count, blood chemistry and computed tomography of the chest and abdomen every 3–6 months. The characteristics of the 67 patients are shown in Table 1. Informed consent was obtained from all patients with respect to performing this analysis.

Extraction of total RNA

Total cellular RNA was extracted using ISOGEN (Nippon Gene, Kanazawa, Japan) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

We carried out real-time polymerase chain reaction (PCR) assays using the ABI PRISM 7700 Sequence Detection System (PE Biosystems, Foster City, CA, USA) to examine chemokine gene expression. For analyzing the expression of IFN-γ, IP-10, I-TAC, RANTES, MIP-1β, IL-4, MDC and eotaxin, pre-developed TaqMan assay reagents (Applied Biosystems, Foster City, CA, USA) specific for each target gene and a control (18S rRNA) were mixed, and the two genes were amplified in the same well (Multiplex Assays) according to the manufacturer's instructions. Threshold values (C_T) were obtained for each sample, and δC_T was calculated by subtracting C_T(control) from C_T(target) for the calibrator and samples. Relative quantification values of target chemokine gene expression of the samples to the calibrator were calculated according to the manufacturer's instructions.

Table 1. Characteristics of the 67 patients with renal cell carcinoma

Mean age ± SE (years)	59.6 ± 12.2
No. men/women	44/23
Mean tumor size ± SE (mm)	60.5 ± 34.1
Stage (*n*)	
I	35
II	0
III	24
IV	8
Grade (*n*)	
I	24
II	35
III	8

For the detection of MIG, primers and a dual-labeled TaqMan probe were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The primers MIGa (5′-TGAGAAAGGGTCGCTGTTCCT-3′) and MIGb (5′-GGCAAATTGTTTAAGGTCTTTCAAG-3′) generate an 83-bp DNA fragment after amplification. The TaqMan probe MIG-P1 (5′-CATCAGCACCAACCAAGGGACTATCCAC-3′), which corresponds to the region from nucleotides 138 to 165, was synthesized and labeled with the fluorescent dyes 6-carboxylfluoroscein on the 5′ end and *N*,*N*,*N*′,*N*′,-tetramethyl-6-carboxyrhodamine on the 3′ end. TaqMan PCR was carried out using an ABI Prism 7700 sequence detection system in duplicate for each sample, in a final volume of 25 μL, according to the manufacturer's instructions. The standard curve for this assay was calculated using five-fold dilutions of a known amount of a calibrator cDNA.

The results from real-time PCR were expressed as the relative expression of target genes normalized to the 18S rRNA endogenous control in the test sample when compared to those in the calibrator.

Antibodies

The antibody used for immunohistochemistry was antihuman CXCR3 monoclonal antibody (49801.111; Genzyme, Cambridge, MA, USA). For flow cytometric analysis, peridinin chlorophyll protein (PerCP)-conjugated antibody to human CD45, and phycoerythrin (PE)-conjugated antibodies to human CD4 and CD8 were purchased from Becton-Dickinson (Mountain View, CA, USA). Fluorescein-isothiocyanate (FITC)-conjugated antibody to human CXCR3 was purchased from Dako (High Wycombe, UK), and FITC-conjugated antibody to human CCR5 was purchased from PharMingen (San Diego, CA, USA).

Analysis of cellular infiltration

Immunocytochemistry was carried out on formalin-fixed and paraffin-embedded tissue sections to analyze cellular infiltration by CXCR3-expressing cells. Pretreatment of sections was done in an autoclave for 20 min at 15 psi and 121°C in target retrieval solution (Dako). The sections were then incubated with 3% H_2O_2 in methanol followed by normal rabbit serum (Dako). The primary antibody was applied at a dilution of 1 : 1000 overnight at 4°C. After washing, the secondary antibody (biotinylated rabbit antimouse immunoglobulin) was applied at a dilution of 1 : 1000. Visualization of the signal was carried out using an SAB staining kit (Dako) according to manufacturer's instructions. CXCR3-positive cells were counted in 10 randomly selected 400× fields (0.22 mm^2/field). The degree of cellular infiltration was shown as the mean number of positive cells ± SE in 10 random fields.

Tumor-infiltrating lymphocyte extraction

Single-cell suspensions from tumor tissue were prepared by mincing the tissue, which was forced through stainless steel mesh into RPMI cell culture medium. The cell suspension was loaded onto a Ficoll gradient and centrifuged at 600*g*. Mononuclear cells were obtained from buffy coats, washed twice with phosphate-buffered saline (PBS), and used for flow cytometric analysis.

Flow cytometric analysis of tumor-infiltrating lymphocytes

Three-color flow cytometry was used to analyze chemokine receptor expression on tumor-infiltrating lymphocytes. Cells (10^6) were incubated with 1 μg of anti-CD45–PerCP, 1 μg of either anti-CXCR3–FITC or anti-CCR5–FITC antibodies, and 1 μg of either anti-CD4-PE or anti-CD8–PE for 30 min at 4°C. For the staining of peripheral blood lymphocytes (PBL), cells were stained in whole blood, then red blood cells were lysed with ethylene glycol. Cells were washed and resuspended in 500 μL of 1% paraformaldehyde in PBS. Analysis was carried out using a Becton Dickinson FACScan with Cell Quest software (Becton Dickinson).

Statistical analysis

The Mann–Whitney *U*-test was used to compare the chemokine expression levels of tumor and normal kidney tissues, and chemokine receptor expression on lymphocytes between PBL and the tumor. Pearson's correlation was used to compare expression levels between genes, and tumor size and the degree of infiltration of CXCR3-positive cells. In order to examine the influence of the level of target gene expression on patient prognosis, Kaplan–Meier analysis was used to compare disease-free survival between the two groups. The patients were divided into two categories according to the level of intratumoral expression. Cut-off was determined to be the mean + 2 SE of the gene expression in normal kidney tissue. The statistical significance was analyzed using the log-rank test. In order to examine the magnitude of contribution of prognostic factors to tumor recurrence, multivariate analysis was carried out using the Cox proportional hazard model. Differences or contributions were considered significant when $P < 0.05$.

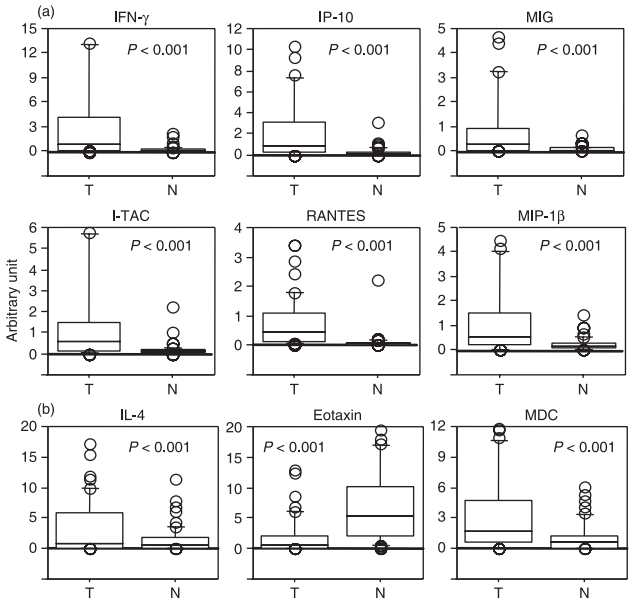

Fig. 1. (a) Expression of Th1-related cytokine, interferon (IFN)-γ and Th1-associated chemokines (IFN-γ inducible protein 10 [IP-10], monokine induced by IFN-γ [MIG], IFN-γ-inducible T cell A chemoattractant [I-TAC], regulated upon activation normal T expressed and secreted [RANTES] and macrophage inflammatory protein [MIP]-1β) determined by quantitative real-time polymerase chain reaction assay. Expression of these genes was significantly increased in tumors (T) when compared to normal kidney tissue (N). (b) Expression of Th2-related cytokine, interleukin (IL)-4 and Th2-associated chemokines (eotaxin and macrophage-derived chemokine [MDC]). The expression of IL-4 and MDC was significantly increased in tumors when compared to normal kidney tissue. In contrast, the expression of eotaxin in the tumor was significantly decreased.

Results

Chemokine gene expression in renal cell carcinoma and normal kidney

Cytokine and chemokine gene expression were analyzed using quantitative real-time PCR to compare expression levels in tumors with those in normal kidney tissue in 67 RCC patients (Fig. 1a,b). IFN-γ and Th1-associated chemokines, including IP-10, MIG, I-TAC, MIP-1β and RANTES, were expressed at significantly higher levels in the tumor than in normal kidney tissue (Fig. 1a). Expression of IL-4 and Th2-associated chemokines, including MDC and eotaxin, were also analyzed. The expression of IL-4 and MDC was significantly higher in the tumor than in normal kidney tissue (Fig. 1b). In contrast, eotaxin expression was significantly decreased in the tumor. These results suggest increased activities of both Th1- and Th2-type immune responses in tumors of RCC.

Chemokine gene expression and tumor factors

We next analyzed the relationship between tumor factors (stage, grade and tumor size) and the level of intratumor expression of the Th1- and Th2-associated chemokine genes. Stage and grade of the tumor showed no relationship to the chemokine expression level (data not shown). Tumor size showed an inverse correlation with the levels of Th1-associated chemokines, including IP-10 (standard regression coefficient $[R] = -0.151$, $P = 0.22$), I-TAC ($R = -0.217$, $P = 0.08$) and MIP-1β ($R = -0.241$, $P = 0.05$), and Th2-associated chemokines, including MDC ($R = -0.133$, $P = 0.28$) and eotaxin ($R = -0.264$, $P = 0.03$), suggesting the possibility of these chemokines as prognostic factors. There was no correlation with MIG ($R = -0.097$, $P = 0.43$) or RANTES ($R = -0.054$, $P = 0.66$).

Correlation between the expression of Th1 and Th2 cytokines and chemokines

In order to examine whether the expression of Th1- and Th2-associated chemokines reflect the activity of the intratumoral immune response, we next analyzed the correlation between the gene expression of IFN-γ and Th1-associated chemokines, and between the expression of IL-4 and Th2-associated chemokines (Fig. 2a,b).

The expression of IFN-γ positively correlated with that of five Th1-associated chemokines (Fig. 2a; data for MIP-1β and RANTES not shown). These correlations were found to be statistically significant. The expression of IL-4 correlated with that of the Th2-associated chemokine eotaxin, but to a lesser extent (Fig. 2b). There was no correlation between the expression of IL-4 and that of MDC, suggesting that the Th1-associated chemokines strongly reflect the activity of the

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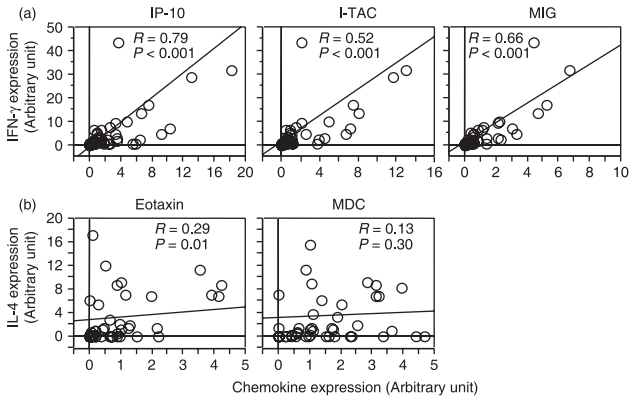

Fig. 2. (a) Expression of interferon (IFN)-γ in the tumor significantly correlates with that of Th1-associated chemokines (IFN-γ inducible protein 10 [IP-10], monokine induced by IFN-γ [MIG] and IFN-γ-inducible T cell A chemoattractant [I-TAC]). (b) Expression of interleukin (IL)-4 in the tumor significantly correlates with that of eotaxin, but not with that of macrophage-derived chemokine (MDC).

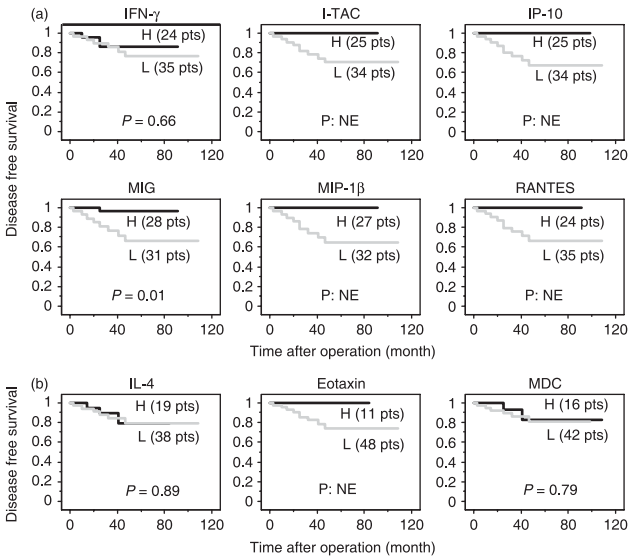

Fig. 3. Kaplan–Meier analysis was used to calculate disease-free survival after curative surgery. Patients were divided into two categories: those with a high level of gene expression (H) and those with low expression (L). Cut-off was set at mean + 2 SE of chemokine gene expression in normal kidney tissue. (a) The expression of interferon (IFN)-γ, IFN-γ inducible protein 10 (IP-10), monokine induced by IFN-γ (MIG), and IFN-γ-inducible T cell A chemoattractant (I-TAC), macrophage inflammatory protein (MIP)-1β and regulated upon activation normal T expressed and secreted (RANTES) were compared. The patients expressing high levels of Th1-associated chemokine rarely experienced tumor recurrence after curative surgery whereas IFN-γ did not influence patient prognosis. (b) The expression of interleukin (IL)-4, eotaxin and macrophage-derived chemokine (MDC) were compared. The expression of IL-4 and MDC did not influence patient prognosis. The statistical influence (*P*-value) of IP-10, I-TAC, MIG, MIP-1β, RANTES and eotaxin could not be evaluated (NE) because one category did not include a recurrence (a statistical event). The statistical difference was analyzed using the log-rank test.

Th1-type immune response. The degree of Th2-type response was reflected by eotaxin to some degree, but not by MDC.

Influence of the expression of Th1- and Th2-associated chemokines on patient prognosis

We next analyzed the influence of Th1- and Th2-associated chemokine expression on patient prognosis. Fifty-nine patients with stage 1–3 tumors underwent curative surgery. The disease-free survival rate was calculated using the Kaplan–Meier method, and compared between the patients expressing high level of target gene and those expressing low level of target gene. The mean follow-up time of these patients was 45.7 ± 29.3 months (2.0–108 months). Nine patients developed recurrence after surgery.

Figure 3a shows the results of Th1-related cytokine, IFN-γ and Th1-associated chemokine, and Fig. 3b shows those of Th2-related cytokine, IL-4 and Th2-associated chemokines. IFN-γ had an influence on patient prognosis (Fig. 3a). In contrast, tumors expressing high levels of Th1-associated chemokine, including IP-10, I-TAC, MIP-1β and RANTES, did not recur after surgery. As there was no recurrent patient in the group expressing higher level of chemokines, the statistical significance was not able to be evaluated. Only one patient expressing high levels of MIG developed a recurrence, and the differences in disease-free survival were statistically significant among groups. The 5-year disease-free survival rate of patients expressing low levels of IP-10, I-TAC, MIG, MIP-1β and RANTES in tumors was approximately 60%. Similar to Th1-associated chemokines, patients expressing high levels of eotaxin were free from tumor recurrence after surgery whereas MDC expression had no influence on patient prognosis. IL-4 did not influence patient prognosis.

To further examine whether the expression level of chemokine genes was an independent factor, we carried out multivariate analysis. Factors that might influence tumor recurrence were analyzed. T stage and intratumor expression of IP-10, I-TAC, RANTES, MIP-1β and eotaxin could not be included in this analysis because the statistical significance could not be calculated. This resulted from that fact that one of the categories in each factor had no patient with tumor recurrence. Thus, the expression of MIG, IFN-γ and IL-4, tumor grade and tumor size were examined. As shown in Table 2, only high expression of MIG was an independent favorable factor.

These results indicate a favorable prognosis of patients with RCC expressing increased levels of Th1-associated chemokines and eotaxin, and their contribution to patient prognosis might be stronger than other clinical factors. In addition, the antitumorigenic activities may depend on the integrity of the immune reaction cascade of the Th1 response, but not on the activities of T helper lymphocytes themselves.

Levels of IP-10, MIG and I-TAC expression correlate with CXCR3-positive cell infiltration into tumors

To examine whether Th1-associated chemokines direct the infiltration of lymphocytes, cellular infiltration of cells expressing CXCR3 was analyzed using immunohistochemistry. Representative images for CXCR3-positive cell infiltration are shown in Fig. 4a,b. CXCR3 was expressed only on the infiltrating cells (Fig. 4c). The average number of CXCR3-

Table 2. Multivariate analysis of prognostic factors related tumor recurrence

Prognostic factor (category)	χ^2	*P*-value	Hazard ratio	95% CI
MIG (high expresser)	6.302	0.012	0.034	0.002–0.475
IFN-γ (high expresser)	1.395	0.237	5.625	0.320–98.892
Tumor size (≤40 mm)	0.972	0.324	0.265	0.019–3.714
IL-4 (high expresser)	0.702	0.402	2.915	0.239–35.600
MDC (high expresser)	0.237	0.626	1.555	0.263–9.206
Tumor grade (grade 1)	0.008	0.972	0.915	0.019–3.714

IFN, interferon; IL, interleukin; MDC, macrophage-derived chemokine; MIG, monokine induced by IFN-γ.

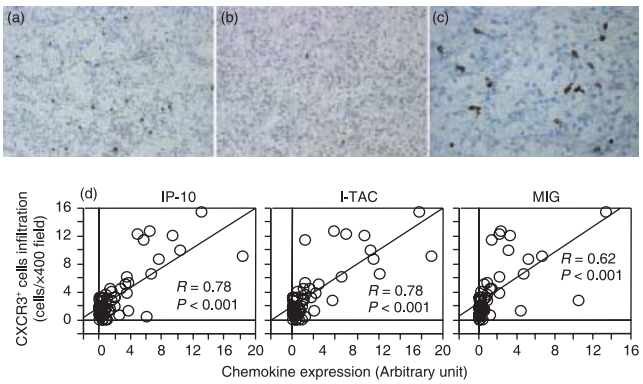

Fig. 4. (a–c) CXC chemokine 3 receptor (CXCR3)-positive cell infiltration into renal cell carcinoma tumor demonstrated immunohistochemically by staining with anti-CXCR3 antibody. The images are representative of tumors expressing (a) high interferon (IFN)-γ inducible protein 10 (IP-10), IFN-γ-inducible T cell A chemoattractant (I-TAC) and monokine induced by IFN-γ (MIG) (×20) and (b) low levels of these chemokines (×20). (c) Observation of the tissue section at high magnification shows that infiltrated cells alone are stained with anti-CXCR3 antibody (×40). (d) The number of CXCR3-positive cells infiltrating the tumor significantly correlated with the expression of IP-10, I-TAC and MIG.

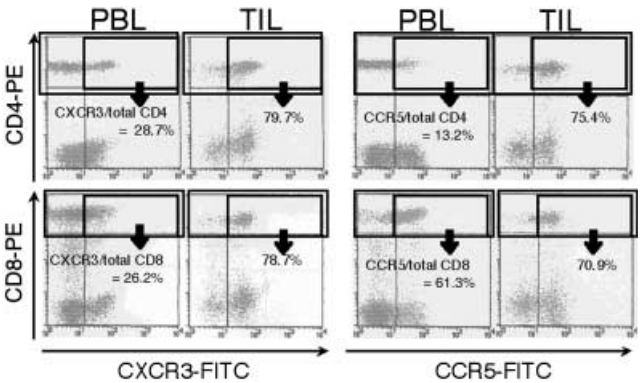

Fig. 5. Expression of the chemokine receptors CXC chemokine 3 receptor (CXCR3) and CC chemokine 5 receptor (CCR5) by three-color flow cytometric analysis in peripheral blood lymphocytes (PBL) and tumor-infiltrating lymphocytes (TIL). The dot plot histogram from the quadrant analysis gated for the expression of CD45 is shown. The majority of $CD4^+$ and $CD8^+$ lymphocytes in the tumor express CXCR3 or CCR5, whereas the proportion of $CD4^+$ and $CD8^+$ lymphocytes expressing chemokine receptors are much lower in PBL. FITC, fluorescein-isothiocyanate; PE, phycoerythrin.

positive cells infiltrating into 400× fields was significantly higher in the tumor (mean 3.5 ± 3.7, range 1–15.5) than in normal kidney tissue (mean 1.1 ± 0.1, range 0.1–4.8). The degree of intratumoral CXCR3-postive cell infiltration correlated positively with levels of IP-10, MIG and I-TAC expression (Fig. 4d). This correlation was statistically significant ($P < 0.01$).

Accumulation of lymphocytes expressing chemokine receptors CXCR3 and CCR5 in the tumors

To examine whether lymphocytes expressing the chemokine receptors accumulated in the tumors, three-color flow cytometric immunophenotyping was carried out. CXCR3 and CCR5 versus CD4 and CD8 expression on gated CD45-positive lymphocytes was examined in 19 patients. Representative results are shown in Fig. 5. The proportion of CXCR3-positive or CCR5-positive cells in total $CD4^+$ or $CD8^+$ cells was calculated. Tumor-infiltrating lymphocytes (TIL) demonstrated a higher proportion of $CXCR3^+CD4^+$ (TIL, 72.7 ± 18.6%; PBL, 26.6 ± 14.9%; $P < 0.001$), $CXCR3^+CD8^+$ (TIL, 70.1 ± 20.3%; PBL, 25.3 ± 17.0%; $P < 0.001$) $CCR5^+CD4^+$ (TIL, 72.4 ± 11.4%; PBL, 19.3 ± 13.1%; $P < 0.001$) and $CCR5^+CD8^+$ (TIL, 77.7 ± 16.0%; PBL, 47.2 ± 18.6; $P < 0.001$) cells than observed in PBL. The majority (more than 70%) of $CD4^+$ or $CD8^+$ T lymphocytes in the tumors expressed CXCR3 or CCR5. These results suggest that the infiltration of type 1 T lymphocytes may be directed by their specific intratumoral Th1-associated chemokines, IP-10, I-TAC and MIG.

Discussion

Chemokines regulate leukocyte migration to the site of inflammation.[1] The chemoattractant properties of some chemokines are preferentially directed to specific lymphocyte populations.[3] TIL are 50–100 times more potent than lymphokine-activated killer cells in mediating regression of established cancer in several murine models.[21] This suggests that chemokines may play an important role in directing TIL trafficking to promote antitumorigenic activities. Experimental models have demonstrated that IP-10 and MIG have shown antitumorigenic activities through $CD4^+$ and $CD8^+$ T lymphocyte recruitment.[22,23] Thus, one possible explanation for the favorable prognosis of patients with increased expression of IP-10, MIG and other chemokines in the tumor is through the ability to direct the recruitment of TIL to the tumor.

Recent studies have shown that some chemokines mediate T lymphocyte recruitment in a selective fashion. The majority of activated T lymphocytes express CXCR3.[19] In addition,

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CXCR3 and CCR5 are preferentially expressed in human Th1 cells.[18] In contrast, Th2 selectively express CCR3 and CCR4.[17,18] Thus, chemokines may influence the efficacy of Th1- or Th2-mediated immune responses in the tumor by selectively recruiting T lymphocytes. These findings raise questions as to what type of immune response is predominant in a favorable RCC tumor and how chemokines are associated with the manifestation of this immune response.

In the present study, we showed increased expression of both IFN-γ and IL-4 genes in tumors compared with normal kidney tissue. Our results are consistent with those from previous reports,[24,25] and suggest that the activities of both Th1 and Th2 are upregulated in RCC tumors. The Th1-type immune response is required for tumor regression.[26,27] Our results also indicate that IP-10, I-TAC and MIG play important roles in recruiting not only type 1 helper T cells, but also effector T lymphocytes into the tumor to polarize and amplify Th1-mediated antitumorigenic cellular immunity in RCC tumors.

Interestingly, our results indicate that the expression of IFN-γ has no influence on patient prognosis whereas the expression of Th1-associated chemokines do. The antitumorigenic activities of the Th1-type immune response may depend on the integrity of the immune reaction cascade, but not on the activities of T helper lymphocytes themselves. The mechanism of chemokine production from the tumor tissue remains to be determined. Our preliminary *in vitro* study has demonstrated the production of IP-10, I-TAC and MIG from RCC cell lines in response to stimulation by IFN-γ, but the amount of chemokine production differs for each cell line (data not shown). Thus, we speculate that the ability of tumor cells to produce Th1-associated chemokines, IP-10, I-TAC and MIG in response to IFN-γ released by type 1 helper T lymphocytes may be one of the determinant factors in integrating the antitumor immune response. An increase in chemokine production induces the recruitment of both helper and effector T cells into the tumor to upregulate antitumorigenic activities. In the present study, MIG was the only factor showing a significant influence on patient prognosis. The other Th1-associated chemokines, including IP-10, I-TAC, MIP-1β and RANTES, could not be included in the statistical analysis because no patient developed recurrence in the group of patients expressing high levels of these chemokines. However, the influence of these chemokines on patient prognosis may be even more important than that of MIG. Thus, we consider Th1-associated chemokines as an important factor to predict patient prognosis.

What we clarified in this manuscript is that the favorable prognosis of RCC patients showing upregulation of Th1-associated chemokine expression may result from the recruitment of TIL to polarize the intratumoral immune response to the Th1-type response. We have not confirmed whether TIL expressing CXCR3 or CCR5 are the effector cells that kill the tumor cells or the helper cells that produce the IFN-γ with the recognition of tumor antigens. This would be the key issue of our hypothesis in which we speculate that chemokines are important mediators in regulating the tumor versus host immune response. To further examine our hypothesis, we need to perform some functional studies *in vitro* to utilize the TIL.

The role of the Th2-type immune response in antitumor immunity remains unclear. Some studies report a suppression of antitumor activity,[26,27] but others do not.[28] A shift in immunological status from Th1 to Th2 in accordance with tumor stage was reported in RCC patients.[29] Our results indicate constitutive upregulation of type 2 helper T cell activity in human RCC. In addition, the increased expression of eotaxin is a favorable factor for patient prognosis. We have not examined the expression of CCR3 or CCR4 in Th2 because of the limitation of reagents at the time when these experiments were carried out. Thus, further investigation is required to clarify the role of the Th2 response.

Conclusions

We showed that most patients with RCC expressing increased levels of IP-10, I-TAC, MIG, MIP-1β and RANTES remain free from recurrence after curative surgery. IP-10, I-TAC and MIG appear to induce the recruitment of Th1 and their effector cells to polarize the intratumoral immune response to IFN-γ-induced antitumor cellular immunity. Integrity of the Th1-type immune response seems to be required for tumor regression. Detection and correction of a defect in the Th1-type response cascade would thus be one of the main targets for tailor-made immunotherapy and gene therapy in RCC.

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