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ORIGINAL ARTICLE

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Combination immunotherapy with OK-432, recombinant granulocyte-colony-stimulating factor and recombinant interleukin-2 for human hepatocellular carcinoma

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Abstract The antitumor effects of immunotherapy using streptococcal preparations (OK-432), recombinant granulocyte-colony-stimulating factor (rG-CSF) and recombinant interleukin-2 (rIL-2) were examined for human hepatocellular carcinoma (HCC). Following subcutaneous injection of OK-432 (2 KE) and rG-CSF (50–60 μg), low-dose intratumoral administration of OK-432 (3–12 KE) was performed. Thereafter, 2×10^5 JRU of rIL-2 was subcutaneously injected. This therapeutic regimen was repeated twice. Serum α-fetoprotein levels were markedly decreased in three of seven patients with HCC by this treatment. Post-therapeutic histological examination revealed that trabecular cords or pseudoglandular arrangements of tumor cells were completely disordered in all cases and that extensive infiltration of lymphocytes into the tumor stroma was present in five cases. The number of CD4- and CD57-positive cells among tumor-infiltrating lymphocytes after immunotherapy was significantly higher than that in patients without immunotherapy ($P < 0.01$). These findings suggest that even a small intratumoral injection of OK-432 can induce extensive infiltration of helper/inducer and natural killer cells into the tumor stroma when combined with subcutaneous injection of OK-432, rG-CSF and rIL-2 and that these cells might play important roles in tumor cytotoxicity.

Key words Hepatocellular carcinoma (HCC) · Immunotherapy · OK-432 (streptococcal preparation) · Recombinant colony-stimulating factor (rG-CSF) · Recombinant interleukin-2 (rIL-2)

Introduction

It has been reported that patients with malignant disease have impaired immunological reactivity [1]. In patients with hepatocellular carcinoma (HCC), decreased natural killer (NK) cell and lymphokine-activated killer (LAK) cell activity has been demonstrated [2, 3]. In order to improve the immunodeficiency of these patients, biological response modifiers (BRM) such as OK-432 (streptococcal preparation [4], recombinant interleukin-2 (rIL-2 [5], interferon (IFN) [6] and other agents have been developed. We previously performed intratumoral injection of OK-432 [7] or rIL-2 [8] in patients with HCC. However, the antitumor effects of OK-432 or rIL-2 alone are not necessarily sufficient therapy for such patients. We have now tested immunotherapy using OK-432, recombinant granulocyte-colony-stimulating factor (rG-CSF) and rIL-2 in experimental hepatic tumors, and we have confirmed that this immunotherapy markedly inhibited tumor growth [9]. We subsequently used this combination therapy for patients with HCC and investigated its antitumor effects.

Materials and methods

Patients

Seven patients with HCC who could undergo hepatic resection and intratumoral injection of OK-432 were enrolled in this study (Table 1). Tumor tissue in each case was pathologically diagnosed as well- to moderately differentiated hepatocellular carcinoma (Edmondson I or II [10]). The patients had not received previous therapy except in case 7. The patient in case 7 had undergone partial hepatectomy for HCC 6 years ago and the recurrence of HCC had been detected. Five patients with HCC who had undergone partial hepatectomy without immunotherapy were selected for a control group.

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Table 1 Clinical and laboratory data on patients. *HCV* Hepatitic *C* virus, *AFP* α-fetoprotein

Case	Age (years)	Sex	Viral marker	Tumor size (mm) (location)	AFP (ng/ml)	Child's grade	Edmondson's classification
1	73	F	Negative	41 × 28 (S8)	4500	A	II
2	58	M	HCV	16 × 14 (S6)	10	A	II
3	59	M	HCV	48 × 47 (S8)	8	A	II
4	59	M	HCV	17 × 15 (S4)	128	B	II
5	58	M	HCV	14 × 12 (S8)	80	B	I
6	57	M	HCV	10 × 8 (S5-8)	212	A	II
7	74	F	HCV	27 × 21 (S3)	60	B	II

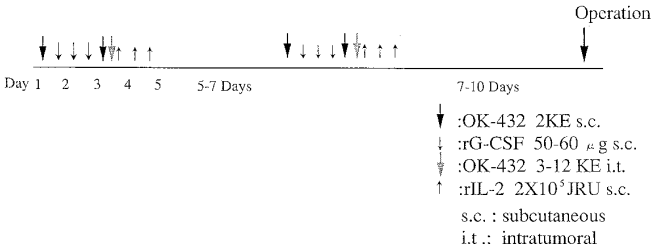

Fig. 1 Therapeutic schedule of combination immunotherapy with OK-432, recombinant granulocyte-colony-stimulating factor (*rG-CSF*) and recombinant interleukin-2 (*rIL-2*)

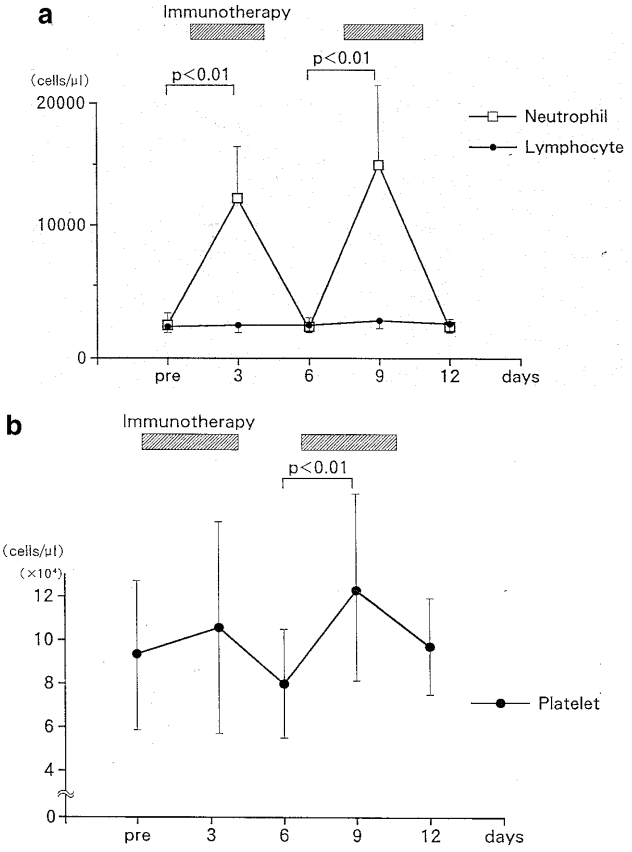

Fig. 2a, b Changes in numbers of (**a**) leukocytes and (**b**) platelets after immunotherapy

Tumor tissue

Tumor tissues before treatment were obtained by aspiration biopsies under ultrasonographic guidance using a Majima needle (21G). Tumor tissues after treatment were harvested by partial hepatectomy. The pre- and post-treatment specimens were fixed in 10% buffered formalin. Specimens were embedded in paraffin, and sections were stained with hematoxylin and eosin (H & E). Histological changes in tumor tissue after immunotherapy were mainly evaluated in two categories: tumor necrosis and infiltration of inflammatory cells into the tumor stroma. The grading of the extent of these changes included five stages, from none to severe.

Immunohistology

Cryostat sections of tumor tissues were cut at a thickness of 5 μm, and the sections were fixed in cold acetone for 10 min. Following air drying, they were immunostained with monoclonal antibodies (mAb) using the avidin-biotin-peroxidase complex method. The mAb employed were mouse anti-human Ig anti-CD4, anti-CD8 (Nichirei) and anti-CD57 (Fujisawa) (diluted × 100). A commercially obtained kit (Histofine, Nichirei) was used for this procedure. The numbers of stained cells were counted in high-power fields (× 400) under a light microscope. The number of positive cells in a section was defined as the cumulative counts for five randomly selected high-power fields.

Administration of drugs

OK-432 and rG-CSF were supplied by Chugai Pharmaceutical Co., Tokyo, Japan. rIL-2 was obtained from Takeda Chemical Industries, Osaka, Japan. After obtaining fully informed consent from each patient, treatment was begun. The schedule of therapy is shown in Fig. 1. Following subcutaneous administration of 2 KE OK-432, 1 μg/kg weight of rG-CSF was subcutaneously injected three times at 12-h intervals. After subcutaneous administration of 2 KE OK-432 again, intratumoral injection of 3–12 KE OK-432 was performed using a PEIT needle under ultrasonographic guidance on the third day of treatment. The dose used for local injection depended on tumor size. In addition, subcutaneous injection of 2×10^5 JRU rIL-2 was performed three times at 12-h intervals. After a 7- to 10-day interval, this therapeutic regimen was repeated once. Partial hepatectomy was then performed 7–10 days after completion of the immunotherapy. Patients had no severe side-effects other than pyrexia.

Statistics

Statistical analyses were performed with the generalized Wilcoxon's test. Differences were considered significant at *P* values less than 0.05.

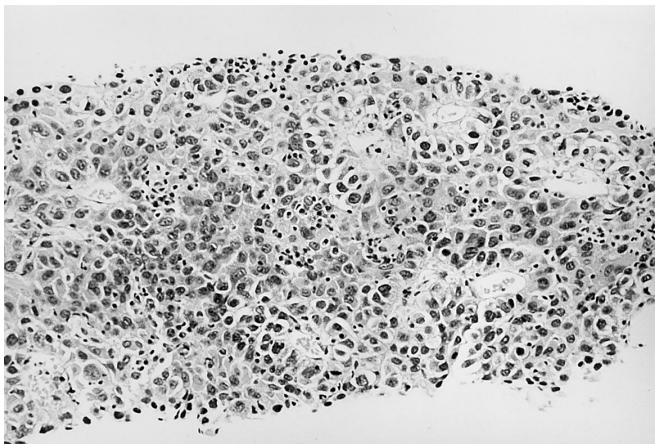

Fig. 3 Histological appearance of hepatocellular carcinoma before immunotherapy in case 1. Many pseudoglandular structures in addition to trabecular cords of tumor cells were seen, and this specimen was therefore diagnosed as Edmondson II. Lymphocyte infiltration was minimal into the tumor stroma. (H&E, original magnification × 100)

Table 2 Changes in serum α-fetoprotein (*AFP*) levels before and after immunotherapy

Case	AFP (ng/ml)	
	Before	After
1	4500	2060
2	10	6
3	8	8
4	128	60
5	80	95
6	212	115
7	60	61

Table 3 Histological appearance after immunotherapy – none, ± very mild, + mild, ++ moderate, +++ severe

Case	Tumor necrosis	Lymphocyte infiltration	Neutrophil infiltration	Plasma cell infiltration
1	±	+++	±	±
2	–	++	±	–
3	–	±	–	–
4	±	+	–	–
5	–	+	±	–
6	–	+	±	±
7	+	±	±	–

Results

Serum α-fetoprotein

Serum α-fetoprotein (AFP) levels were assessed before and 3–5 days after immunotherapy. The levels in cases 2 and 3 before treatment were within the normal range. Changes in serum AFP levels before and after immunotherapy are shown in Table 2. AFP values were markedly decreased to half the pre-treatment values in three of seven patients

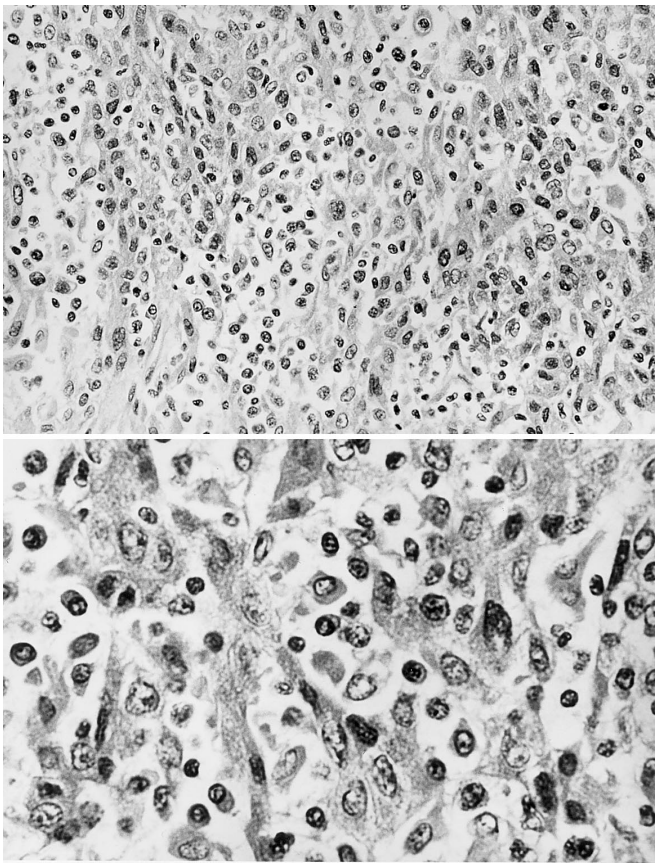

Fig. 4a, b Histological findings of hepatocellular carcinoma after treatment in case 1. **a** Pseudoglandular structures and trabecular cords were disordered, and severe infiltration of lymphocytes into the tumor stroma was observed. Infiltration of neutrophils into the tumor tissue was less pronounced than that of lymphocytes. Tumor necrosis was not observed (H&E, original magnification × 100). **b** Most tumor-infiltrating lymphocytes were moderate- to large-sized. Plasma cell infiltration was observed in a part of tumor tissue. (H&E, original magnification × 400)

treated with this combination therapy. However, the rate of reduction in AFP level was not correlated with tumor size. In case 5, the AFP level was higher after treatment than before, because another HCC lesion was present under the dome of the liver, where local administration of OK-432 could not be performed.

Changes in peripheral blood cell counts

The number of neutrophils in peripheral blood was transiently increased after subcutaneous injection of OK-432 and rG-CSF (1,790±859 compared to 12,256±4,318, $P < 0.01$) (Fig. 2). However, there was no correlation between the increase in the number of neutrophils and the decrease in serum AFP level (data not shown). The number of neutrophils returned to the pre-treatment levels after completion of immunotherapy. On the other hand, lymphocytes in peripheral blood did not significantly change in number during immunotherapy. The change in number of

Table 4 Comparison of tumor-infiltrating lymphocyte subpopulations in groups treated with and without immunotherapy. Values are cumultive counts for five high-power fields (× 400)
* $P < 0.05$
** $P < 0.01$

Group	Anti-CD4	Anti-CD8	Anti-CD57
Immunotherapy (n = 7)	81.6 ± 55.2 **	19.0 ± 9.0 *	79.7 ± 67.2 **
Control (n = 5)	14.6 ± 14.4 **	9.4 ± 10.6	16.0 ± 5.8 **

platelets during immunotherapy was similar to that of neutrophils. The number of red blood cells remained at pre-treatment levels throughout treatment. During the second course of the immunotherapy, the numbers of peripheral blood cells were about the same as those during the first course.

Histological findings

Some lymphocytes were scattered in the tumor stroma before immunotherapy (Fig. 3). After treatment, trabecular cords or pseudoglandular arrangements composed of neoplastic cells were completely disordered in all cases. A somewhat greater number of tumor-infiltrating lymphocytes (TIL) were found in the tumor stroma in two cases. Moderate TIL levels were observed in three cases (Table 3). Inflammatory cells infiltrating the tumor stroma were mostly moderate- to large-sized lymphocytes (Fig. 4). In two of seven cases, plasma cells were observed in a part of the tumor tissues (Fig. 4). However, the extent of infiltration of neutrophils into tumor stroma was smaller than that of lymphocytes in all cases. On the other hand, tumor necrosis was generally minimal in all patients.

Immunohistochemical staining

Since most of the inflammatory cells in the tumor tissues after immunotherapy were lymphocytes, the phenotype of the TIL was examined using immunohistochemical staining. The number of CD4-positive T cells was significantly higher than that of CD8-positive T cells (81.6 ± 55.2 compared to 19.0 ± 9.0, $P < 0.05$).

Compared with the patients who did not undergo immunotherapy, those who did had significantly more CD4-positive (79.7 ± 67.2 compared to 16.0 ± 5.8, $P < 0.01$) and CD57-positive (81.6 ± 55.2 compared to 14.6 ± 14.4, $P < 0.01$) cells in their TIL (Table 4).

Discussion

It has previously been found that OK-432 not only has a direct cytostatic effect on tumor cells [11] but that it also indirectly augments the actions of effector cells including NK cells [12, 13], LAK cells [14], cytotoxic T lymphocytes [15], macrophages [16] and other types of cells in tumor tissues. Therefore, local injection of a large amount of OK-432 has been used to treat malignant tumors [17, 18]. The efficacy of this treatment was, however, less than expected. Subsequently it was reported that combined immunotherapy with OK-432 and cytokines such as rIL-2 [19, 20] or IFN [21] had enhanced antitumor effects.

Recently, rG-CSF [22], which not only increases the number of neutrophils but also activates their function, has been developed. On the other hand, previous studies showed that OK-432-induced neutrophils might play important roles in the cytotoxicity of tumor cells in ascites [23]. In order to augment the antitumor effects of neutrophils, some investigators have designed combination therapies with OK-432 and rG-CSF for tumor-bearing experimental animals [24–26]. They confirmed that the methods and timing of administration of these agents are very important for generating stronger antitumor effects. We therefore tested a new immunotherapy with OK-432, rG-CSF and rIL-2 in patients with HCC using Saito's protocol [26].

In the present study, extensive infiltration of lymphocytes into the tumor stroma was observed in most patients whose serum AFP levels were markedly decreased (Tables 2, 3). However, the extent of lymphocyte infiltration after treatment did not depend on tumor size.

The TIL phenotype was also analyzed. Since T cell were predominant among TIL [27], we used anti-CD-4 and CD-8 as monoclonal antibodies and found that the the number of the helper/inducer T cells was significantly higher than that of cytotoxic/suppressor cells in TILs after immunotherapy (Table 4).

It was also shown that the tumor tissues treated with immunotherapy had significantly more extensive infiltration of helper/inducer T lymphocytes and NK cells into the tumor stroma than those without this treatment. Moreover, TIL after treatment were mainly moderate- to large-sized lymphocytes, indicating that lymphocytes blastformation had occurred [28]. The phenomena described above were not observed following local injection of approximately the same dose of OK-432 alone (data not shown). Thus, even low-dose intratumoral injection of OK-432 could induce extensive infiltration of lymphocytes into the tumor stroma when combined with subcutaneous injection of OK-432, rG-CSF and rIL-2.

Unexpectedly, infiltration of neutrophils into the tumor stroma was less extensive than that of lymphocytes. These findings suggest that combined immunotherapy with OK-432, rG-CSF and rIL-2 may have stimulated lymphocyte-mediated immune responses rather than neutrophil-mediated immune responses [26].

The fact is that the number of neutrophils infiltrating the experimental hepatic tumor was much higher than that of lymphocytes [9]. This finding differed from those for human HCC. However, the dose and means of administration of these agents were slightly different for experimental hepatic tumor and human HCC. In addition, the interval

from completion of immunotherapy to obtaining tumor tissues also differed. The cell line used for hepatic tumors was colon 26, which is histologically adenocarcinoma [29]. Further studies will be needed to determine the differences between experimental hepatic tumor and human HCC.

On the other hand, the tumor necrosis induced by this treatment was generally minimal. One of the reasons for minimal necrosis may be that the amount of local administration of OK-432 was very small. In brief, it was suggested that a direct effect of OK-432 was not enough to induce tumor necrosis. Intratumoral injection of a large amount of OK-432 might result in widespread tumor necrosis [11, 12].

In summary, combined immunotherapy with OK-432, rG-CSF and rIL-2 in patients with HCC exerted antitumor effects through inflammatory cells, most of which were lymphocytes. This treatment should be considered one of the useful therapeis for patients with HCC.

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