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Enhancement of Therapeutic Effect of Interleukin-2 on Spontaneous Pulmonary Metastases of Lewis Lung Carcinoma by Killer Helper Factor Associated with Increased Induction of Killer Activity

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Killer helper factor (KHF) was previously found to be produced by a human T cell hybridoma, 24A • CA2. We studied the therapeutic effects of interleukin-2 (IL-2) and KHF on the inhibition of pulmonary metastases of syngeneic Lewis lung carcinoma (3LL) in C57BL/6N mice. Multiple subcutaneous (sc) injections of IL-2 plus KHF had significantly more effect than injections of IL-2 alone in inhibiting spontaneous pulmonary metastases and prolonging survival of the mice. The effect of KHF with IL-2 on induction of lymphokine(IL-2)-activated killer (LAK) activity against P-29 cells was examined in the murine system. Spleen cells generated LAK activity after incubation for 4 days with more than 500 U/ml of IL-2. In contrast, KHF alone did not render spleen cells cytotoxic. The combination of these lymphokines at subthreshold concentrations, however, resulted in significant *in vitro* induction of LAK activity. The LAK activity of splenocytes incubated with IL-2 plus KHF was maximal after 4 days, and persisted for longer than that of cells treated with IL-2 alone. The LAK cells induced by KHF plus IL-2 were also cytotoxic to FBL and YAC-1 cells. Moreover, spleen cells of mice bearing lung metastases could be induced to the cytotoxic state by sc injections of IL-2 plus KHF. These results indicate that combination treatment with IL-2 and the new lymphokine KHF should be useful clinically in inducing LAK activity for inhibition of pulmonary metastases.

Key words: Lung metastasis — Interleukin-2 — Killer helper factor — LAK induction

Interleukin-2 (IL-2) has been shown to have anti-metastatic activity in animals[1-3] and preliminary clinical trials have shown that it also has this activity in humans.[4-6] In previous studies in animals, however, this antitumor activity was observed either with high doses of IL-2 alone[2] or with lower doses of IL-2 in combination with adoptive transfer of lymphokine(IL-2)-activated killer (LAK) cells.[2,7] IL-2 was found to mediate various effects on cytotoxic lymphoid cells, such as induction of LAK activity[8] and augmentation of NK-cell activity.[9,10] But, systemic administration of high doses of IL-2 alone does not seem to be effective for induction of the cytotoxic cells[11] that are responsible for eradication of malignant cells, because injected IL-2 is rapidly cleared from the blood.[12] Moreover, systemic administration of IL-2 is associated with undesirable side effects, such as pulmonary edema due to enhanced permeability.[13] There are several reports that in murine tumor models IL-2 in combination with other biological response modifiers (BRM), such as interferon (IFN)-α and tumor necrosis factor (TNF), is more effective in induction of cytotoxic activity with anti-metastatic activity than either IL-2 or a BRM singly.[14-16]

A new lymphokine, KHF, with IL-1-like activity (MW, 20,000) was recently found to mediate killer helper activity in the induction of cytotoxic T cells.[17] Killer helper factor (KHF) activates T cells, inducing productions of IL-2, B cell differentiation factor and IFN-α.[17] IL-1 is also known to induce IL-2 receptor expression and production of IL-2 by T cells,[18,19] but Northern blot analysis showed that mRNA from a T cell hybrid clone producing KHF did not cross-react with cDNA for human IL-1 (β).[17] As both KHF and IL-2 singly potentiate T cell-mediated anti-tumor activity, it seemed possible that in combination they might have synergistic effects on generation of LAK activity for host defense. For example, combination therapy with KHF and IL-2 might be effective in conditions in which IL-2-activated killer cells are the main effector cells against primary and/or metastatic tumors. With this combination therapy it might also be possible to reduce the effective dose of IL-2 and so avoid the severe side effects associated with high doses of IL-2.

In the present study we examined the *in vivo* anti-metastatic effects of combinations of KHF and IL-2 using a murine 3LL lung metastasis model. We found that KHF enhanced the therapeutic effect of IL-2 and that the combination was more effective than either lymphokine alone against pulmonary micrometastases. We also showed that this antimetastatic effect was associated with induction of LAK activity in the spleen.

MATERIALS AND METHODS

Animals Specific pathogen-free male C57BL/6N mice of 8–10 weeks old were obtained from Shizuoka Experimental Animal Farm (Shizuoka).

Tumor The 3LL tumor, an undifferentiated squamous cell carcinoma that arose spontaneously in the lung of a C57BL/6N mouse, has been maintained by serial biweekly sc passage in the same strain of mice.[20] A local tumor grown in the back was removed and minced aseptically. The tumor fragments were stirred in RPMI 1640 containing 0.2% trypsin (1:25; Difco Laboratories, Inc., Detroit, Mich.) at 37°C for 30 min. The isolated tumor cells were washed twice with RPMI 1640 containing 10% fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N.Y.), resuspended in fresh RPMI 1640, and counted in a hemocytometer. The viability of tumor cells was estimated as more than 90% by the trypan blue dye exclusion test. A suspension of 10^6 viable cells in 0.05 ml of RPMI 1640 was implanted sc into the hind footpad of each mouse. P-29 variant cells subcloned from 3LL parent cells to be adapted to grow in culture, were a gift from Dr. Keizo Takenaga, Chiba Cancer Center, Chiba.[21] Friend virus-induced leukemia (FBL) and Moloney virus-induced leukemia (YAC-1) were obtained from Osaka University. These cell lines were maintained as single cell suspensions.

Reagents Recombinant human IL-2 (specific activity, 5×10^7 U/1.11 mg/ml) was a gift from Ajinomoto Co. Ltd., Kawasaki. IL-2 was resuspended in RPMI 1640 containing 0.1% mouse albumin (Sigma Chemical Co., St. Louis, Mo.) and frozen until use. KHF was purified from cultures of hybrid cells, 24A·CA2.[17] Briefly, the supernatant of cultures of human T hybridoma cells stimulated for 48 h with PMA was fractionated by gel filtration on Sephadex G-100 in 0.5 *M* NaCl, and the fractions of 15,000 to 25,000 daltons (20 kd sup) were collected, dialyzed against 0.01 *M* Tris-HCl buffer, pH 7.6, and subjected to ion-exchange chromatography. The fraction of MW 20,000, designated as KHF,[17] was used in the present studies.

Preparation of effector cells As described previously,[22] the spleen was transferred aseptically to RPMI 1640 and gently crushed with the flat end of a sterile syringe. The cells were filtered through a stainless steel mesh and washed briefly with distilled water to lyse erythrocytes. The splenocytes were then washed three times with RPMI 1640, resuspended in RPMI 1640 supplemented with 5% FBS, and incubated at various cell densities with or without IL-2 for 4 days, unless otherwise mentioned.

Determination of LAK activity LAK activity was assayed by measuring ^{51}Cr-release from P-29 cells.[23] Briefly, splenocytes were thoroughly washed twice and their cytotoxicity against 1×10^4 ^{51}Cr-labeled P-29 cells was measured. Incubations were terminated after 4 h, and the supernatants (0.1 ml per well) were collected by brief centrifugation at 1,500 rpm, and their radioactivities were determined in a gamma counter. Percentage cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = 100\times\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}$$

The spontaneous release observed with P-29 target cells ranged from 5 to 15% (total % lysis).

Spontaneous pulmonary metastasis system Mice with established spontaneous pulmonary and lymph node metastases of Lewis lung carcinoma (3LL) cells were used in therapeutic studies.[20, 22] For production of spontaneous metastases, 1×10^6 viable 3LL tumor cells in 0.05 ml of RPMI 1640 were injected sc into one hind footpad of each mouse. After 12 days, when the tumor had grown and produced pulmonary metastases, the tumor-bearing leg was amputated at the midfemur by the cautery clamp technique. The edge of the wound was then closed with Michel clips under aseptic conditions. Mice were autopsied 12 days after removal of the implanted tumor, and pulmonary metastases were estimated grossly by fixing the lung in 10% formaldehyde solution and counting the numbers of metastatic nodules on the pulmonary surface. In a second set of experiments, mice were observed daily for 30 days. Dead or moribund mice were necropsied and examined for the presence of disseminated cancer.

Statistical analysis Statistical significance was assessed by using Student's *t* test (2-tailed) or the Mann-Whitney *U* test.

RESULTS

***In vitro* induction of LAK activity in the spleen of normal mice** First we examined whether KHF and IL-2 had synergistic effects in *in vitro* induction of LAK activity of spleen cells of C57BL/6N mice. For this, the spleen cells were incubated for 4 days in medium with or without IL-2 (500 or 5000 U/ml) and with or without various amounts of KHF, and then LAK activity against P-29 cells was measured. As shown in Table I, KHF alone at concentrations of up to 0.5 U/ml did not induce LAK activity in spleen cells, while IL-2 (500 or 5000 U/ml) induced significant LAK activity, and a combination of IL-2 and KHF ($\geqq$0.05 U/ml) induced significantly more LAK activity than IL-2 alone.

We also examined the kinetics of the effects of IL-2 and KHF in induction of LAK activity. Splenocytes with or without IL-2 (5000 U/ml) were incubated in medium with or without KHF (0.5 U/ml) for the times indicated

Table I. Effects of IL-2 and KHF on Induction of LAK Activity

Concentration of KHF (U/ml)	Percent cytotoxicity against P-29 cells[a] IL-2 concentration (U/ml) 0	500	5000
0	0	23.6±3.9[b]	41.5±5.1
0.005	0.3±0.4	24.1±2.6	42.1±2.5
0.01	0	29.8±2.2	45.7±2.6
0.05	0.8±0.6	32.3±1.2[c]	48.9±3.2
0.1	0.4±0.6	37.0±4.2[c]	57.5±2.2[c]
0.5	3.5±1.6	42.2±4.8[c]	75.2±1.2[c]
1	11.4±0.3	49.3±3.9[c]	81.2±1.6[c]

a) Spleen cells (5×10^5) were incubated for 4 days in medium alone or with IL-2 (500 or 5000 U/ml) with or without the indicated concentrations of KHF. Then their LAK activity against 1×10^4 ^{51}Cr-labeled P-29 cells was measured as described in "Materials and Methods."
b) Mean±SD for triplicate cultures.
c) Significantly different from the value for splenocytes treated with IL-2 alone ($P<0.05$).

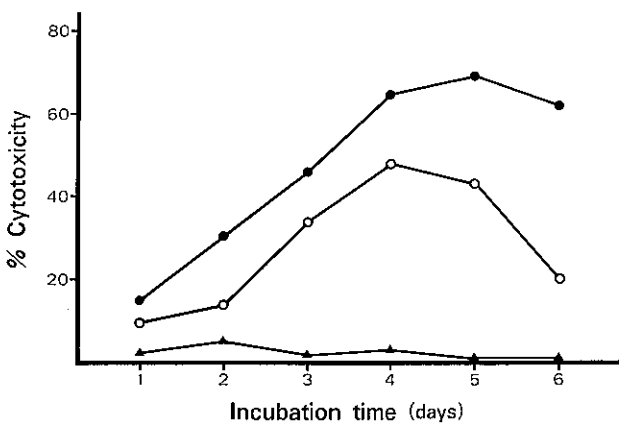

Fig. 1. Effect of KHF on *in vitro* LAK induction by IL-2. Spleen cells were incubated for the indicated periods in medium containing 5000 U/ml of IL-2 with or without 0.5 U/ml of KHF. Then their LAK activity on 10^4 ^{51}Cr-labeled P-29 cells was assayed at an E/T ratio of 50:1. ●, KHF+IL-2; ○, IL-2 alone; ▲, KHF alone. Data are representative of three separate experiments. SDs were consistently <10% of means.

in Fig. 1. Splenocytes showed no LAK activity when incubated for up to 6 days with KHF alone. LAK activity induced by IL-2 alone was maximal on day 4 of incubation, and then decreased gradually. KHF plus IL-2 induced significantly more LAK activity than IL-2 alone; the induction continued for 5 or 6 days and was sustained at a higher level than that induced by IL-2 alone. We also examined the effect of KHF on the

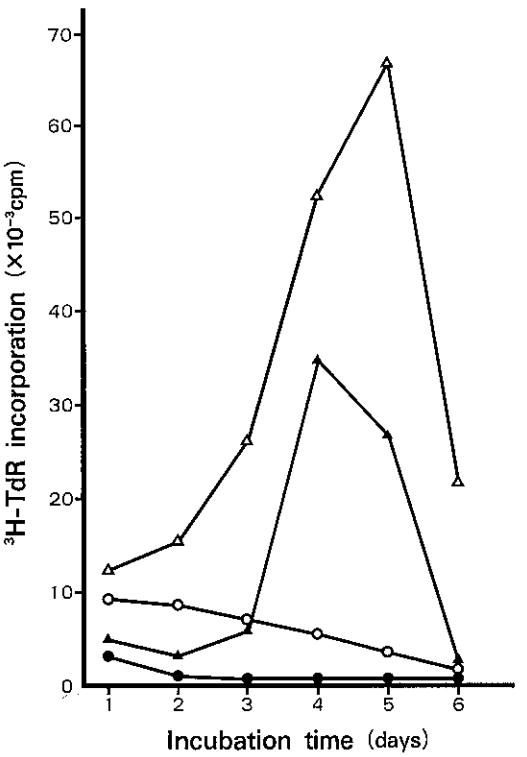

Fig. 2. Effect of KHF on the *in vitro* proliferative response of spleen cells to exogenous IL-2. Spleen cells were incubated for the indicated periods in medium containing 5000 U/ml of IL-2 with or without 0.5 U/ml of KHF and pulsed with ^{3}H-TdR 14 h before harvesting. △, KHF+IL-2; ▲, IL-2 alone; ○, KHF alone; ●, medium alone. Data are representative of 3 separate experiments. SDs were consistently <10% of means.

blastogenic response of spleen cells to IL-2 under the same experimental conditions by measuring uptake of ^{3}H-TdR by the splenocytes (Fig. 2). Splenocytes showed little proliferation in response to KHF. IL-2 stimulated the blastogenic response of splenocytes time-dependently with a maximum on day 4, followed by a rapid decrease. IL-2 plus KHF induced a significantly greater proliferative response than IL-2 alone (Fig. 2).

LAK activity of splenocytes induced by IL-2 and KHF *in vitro* The LAK activity of splenocytes induced by IL-2 (5000 U/ml) *in vitro* was significantly cytotoxic to 3 different murine tumors: P-29, FBL and YAC-1 (Table II). IL-2 (500 U/ml) alone did not activate splenocytes, but IL-2 plus KHF (0.5 U/ml) induced marked LAK activity against all three murine tumors.

Induction of LAK activity of splenocytes of tumor-bearing mice For examination of the effect of pulmonary metastasis on *in vitro* induction of LAK activity of splenocytes, mice bearing pulmonary metastases were

killed on day 1, 3 or 5 after surgical removal of the primary sc tumor. Then their splenocytes were harvested and incubated in medium with or without IL-2 and/or KHF for 4 days and tested for LAK activity against P-29 cells. As shown in Table III, these splenocytes of mice bearing pulmonary metastases expressed LAK activity in response to IL-2, and significantly more in response to KHF plus IL-2. There was no difference in the expressions of LAK activity by spleen cells of normal and tumor-bearing mice in response to IL-2 and/or KHF.

***In vivo* induction of LAK activity in the spleen of mice bearing pulmonary metastases** The above data demonstrated that splenocytes of tumor-bearing mice expressed LAK activity after incubation with IL-2 and KHF *in vitro*. We next examined the more significant question of whether splenocytes of mice bearing lung metastases expressed LAK activity after treatment with KHF plus IL-2 *in vivo*. For this, mice bearing pulmonary metastases were treated with 1 U/mouse of KHF and 5×10^4 or 10×10^4 U/mouse of IL-2 three times a day at 8-h intervals on days 13–16 after tumor implantation. On day 17 their splenocytes were isolated and tested for LAK activity on P-29 target cells. As shown in Fig. 3, these treatments induced significant LAK activity of splenocytes of mice bearing pulmonary metastases, and KHF (1 U/mouse) plus IL-2 (10×10^4 U/mouse) had more effect than IL-2 alone.

Inhibition of spontaneous pulmonary metastases by KHF and IL-2 Spontaneous pulmonary metastases were produced in C57BL/6N mice by sc injection of 10^6 3LL cells

Table II. Effect of LAK Activities Induced by IL-2 and KHF on Various Murine Tumors

Treatment[a]		Percent cytotoxicity against		
KHF	IL-2(U/ml)	P-29	FBL	YAC-1
−	0	0	0	0
+	0	3.4±1.2	0	0
−	500	9.8±2.1[b]	5.2±1.2	−2.5±0.9
+	500	51.2±2.8[c]	45.4±4.3[c]	24.4±0.5[c]
−	5000	42.1±3.7	35.0±2.8	25.4±2.5
+	5000	87.0±4.4[c]	85.1±1.3[c]	69.1±3.8[c]

a) Spleen cells were incubated for 4 days in mediun containing IL-2 (500 or 5000 U/ml) with or without KHF (0.5 U/ml). Then their LAK activity against the indicated target cells prelabeled with ^{51}Cr was measured.
b) Mean±SD for triplicate cultures. Data are representative of two separate experiments.
c) Significantly different from the value for spleen cells incubated in medium with IL-2 alone at the corresponding concentration ($P<0.05$).

Table III. Induction of LAK Activity of Splenocytes of Tumor-bearing Mice

Treatment[a]		Days after removal of tumor		
KHF	IL-2(U/ml)	1	3	5
−	0	0±0.2[b]	2.3±0.7	3.2±1.2
+	0	1.2±0.7	3.9±0.6	6.8±3.5
−	5000	45.8±2.4	60.4±2.7	62.3±5.4
+	5000	64.9±4.9[c]	74.9±4.2[c]	78.5±4.9[c]

a) Mice bearing pulmonary metastases were killed on day 1, 3 or 5 after surgical removal of the primary tumor. Their splenocytes were isolated and incubated for 4 days in medium containing IL-2 (5000 U/ml) with or without KHF (0.5 U/ml). Then LAK activity of the splenocytes was assayed.
b) Mean±SD for triplicate cultures.
c) Significantly different from the value for spleen cells incubated in medium with IL-2 alone at the corresponding concentration ($P<0.05$).

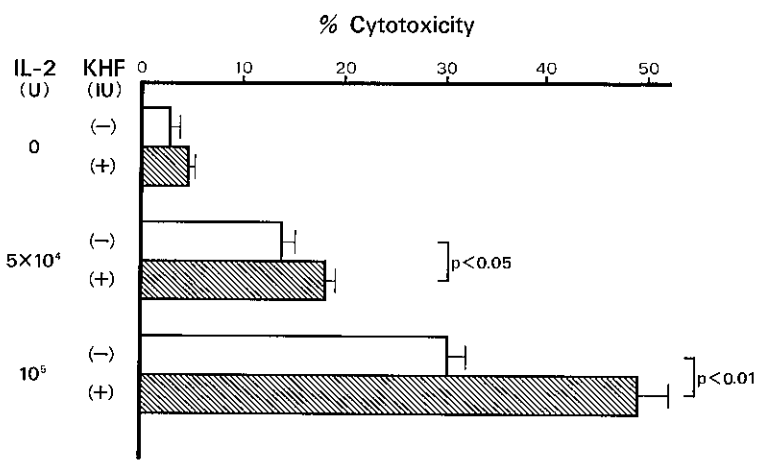

Fig. 3. Effect of combined therapy with KHF and IL-2 on development of LAK activity in spleen cells of mice with pulmonary metastases. 10^6 3LL cells were injected sc into the footpads of groups of 3 mice. Primary tumors were removed on day 12 after implantation. KHF (1 U) and IL-2 (5×10^4 or 10×10^4 U) were injected sc on days 13, 14, 15 and 16. Spleen cells were collected on day 17 and their LAK activity on 1×10^4 labeled P-29 cells was tested at the E/T ratio of 50:1. Columns show representative results in 3 separate experiments. Bars show SDs of means.

into a footpad. Twelve days later when spontaneous pulmonary micrometastases had been established,[20, 22] the foot with the growing tumor was amputated. In the first experiment, daily sc injections of KHF (1 U/mouse) and/or IL-2 (10^5 U/mouse) were given from 24 h after amputation of the foot. All mice were killed 24 days after the sc inoculation of tumor cells. As shown in Fig. 4, sc injections of IL-2 (10^5 U/mouse) alone significantly inhibited development of spontaneous pulmonary metastases of 3LL cells, whereas injections of KHF alone had no effect. Treatment with KHF (1 U/mouse) plus IL-2 (10^5 U/mouse) was significantly more effective than treatment with IL-2 alone (Fig. 4). Thus, the inhibitory effect of IL-2 on pulmonary metastases was enhanced by simultaneous treatment with KHF.

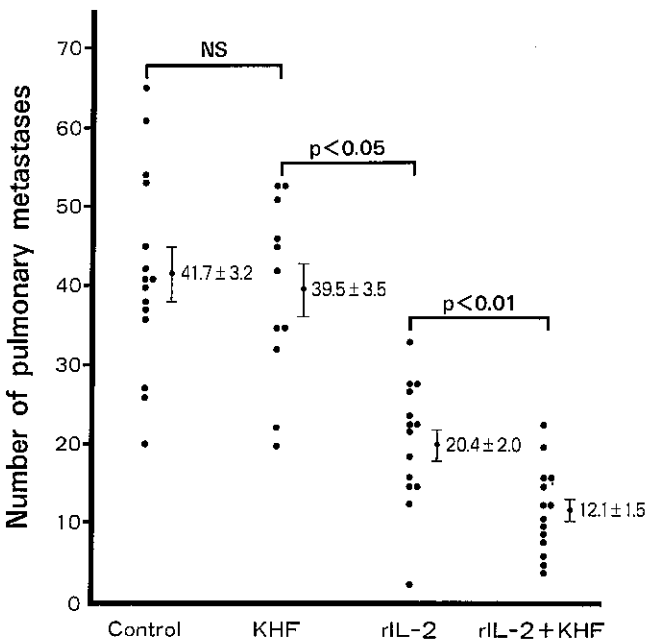

Fig. 4. Inhibition of spontaneous pulmonary metastases by KHF and/or IL-2. 10^6 3LL cells were injected sc into the footpad. Primary tumors were removed on day 12 after implantation. KHF (1 U/mouse) and/or IL-2 (10^5 U/mouse) were injected sc three times a day from day 13 to day 19. Mice were killed on day 24 and pulmonary metastases were counted. Points and bars show means±SE of numbers of surface nodules. Data are representative of 3 separate experiments. Statistical significance was assessed by Student's *t* test.

Effects of KHF and/or IL-2 on the survival of mice with lung metastases The effects of KHF and/or IL-2 on survival of mice with pulmonary metastases are shown in Fig. 5. Daily sc injections of KHF (1 U/mouse) and/or IL-2 (10^5 U/mouse) were given three times a day from day 10 to day 16 after tumor implantation, and mice were observed daily until day 30. Daily sc injections of IL-2 significantly prolonged the survival of these mice and KHF plus IL-2 was significantly more effective than IL-2 alone ($P<0.01$).

DISCUSSION

In the present study we demonstrate that combination therapy with KHF and IL-2 was far more effective in both eradication of spontaneous pulmonary metastases of 3LL cells and prolonging the survival of the mice with metastases than IL-2 alone. We also found that KHF enhanced induction of LAK activity of splenocytes by IL-2 both *in vitro* and *in vivo*.

The factor designated as KHF, produced by human T cell hybridomas, was previously found to augment the induction of cytotoxic T cells against ultraviolet-treated stimulator cells in primary mixed lymphocyte culture.[17] Moreover, KHF augmented not only the production of IL-2 and other lymphokines by T cells but also the expression of IL-2 receptors. IL-1 also facilitates the production of IL-2 and the expression of IL-2 receptor,[24, 25] but no mRNA for IL-1β was detectable by Northern blot analysis with cDNA for human monocyte-derived IL-1β in a stimulated T cell hybridoma (24A · CA2) that secreted KHF.[1] Moreover, in preliminary studies KHF appeared to be distinct from IL-1α, IL-4 and IL-6 because their cDNAs showed no cross-

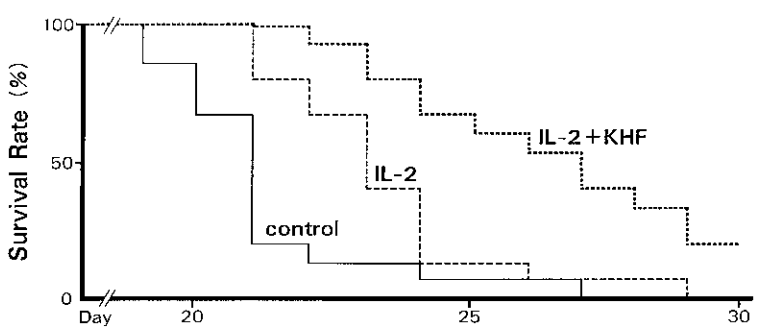

Fig. 5. Combined effect of KHF and IL-2 on the survival of mice with pulmonary metastases. 10^6 3LL cells were injected sc into a footpad. The implanted tumor was removed on day 9 after tumor implantation. KHF (1 U/mouse) and/or IL-2 (10^5 U/mouse) was injected sc three times a day from day 10 to day 16 and the mice were observed daily until day 30. ·····, KHF+IL-2; ----, IL-2 alone; ——, RPMI 1640 alone.

hybridization. In the present work we found that KHF alone did not induce significant LAK activity *in vitro* or *in vivo* (Fig. 2). This observation, together with a previous finding that KHF shows no IL-2 activity,[17] indicates that KHF is a new lymphokine distinct from IL-2. This conclusion is also supported by evidence for possible production of such a factor in models using a murine T hybridoma,[26-28] murine splenocytes,[29] a cloned thymic epithelial cell line[30] and an HTLV-1-transformed human T cell line.[31] Molecular cloning is necessary to determine whether the factor(s) in the other systems is identical with KHF, or is another cytokine with similar functions. Studies are in progress on this problem.

Accumulating evidence that induction of LAK cells by IL-2 is important in host defense against primary and/or metastatic neoplasias has stimulated interest in agents that can increase LAK-mediated destruction of tumor cells *in vivo*. For example, there are several reports showing that induction of LAK activity by IL-2 can be enhanced by cytokines such as IFN-α[14] or TNFα[15] or bacterial preparations such as *Nocardia rubra* cell wall skeleton.[32] In this study we found that KHF significantly enhanced the induction of LAK activity by IL-2 *in vitro* and *in vivo*, suggesting that it acts as an immunoregulatory molecule in induction of LAK activity by IL-2. However, the mechanism of the enhancing effect of KHF on induction by IL-2 of the LAK activity responsible for eradication of pulmonary metastases is still unknown. As KHF alone did not induce LAK activity *in vitro* or *in vivo*, its induction of humoral factors or cellular events may contribute to its enhancement of LAK induction by IL-2 *in vitro* and *in vivo*. The precursors of LAK cells are known to be heterogeneous lymphoid cell subpopulations consisting of NK cells and T cells.[10, 33-35] KHF may stimulate endogenous production of IL-2, which activates T cells to produce the IFN-γ responsible for expression of IL-2 receptors.[17] These present findings, together with previous observations indicating that KHF may augment the generation of cytotoxic T lymphocytes with production of lymphokines (IL-2 and IFN-γ) in murine systems,[36] suggest that LAK cells induced from both T cells and NK cells by the action of KHF plus IL-2 may be involved in destruction of pulmonary metastases. The mechanism by which KHF and IL-2 in combination eradicated pulmonary micrometastases *in vivo* probably involves LAK cells, because in this study we found the anti-metastatic activity of the two cytokines was correlated with the generation of LAK activity in spleen cells.

The present study showed that multiple sc injections of high doses of IL-2 alone reduced established pulmonary metastases of syngeneic 3LL lung carcinoma, but that KHF alone was not effective. Moreover, treatment of mice bearing micrometastases with KHF plus a suboptimal dose of IL-2 was significantly effective in inhibiting spontaneous pulmonary metastases and prolonging the survival of the mice. This synergism of KHF and IL-2 should be useful clinically, because in combination with KHF the dose of IL-2 required to induce LAK activity *in vivo* can be reduced. Thus, clinical use of the two lymphokines (KHF and IL-2) in combination should be effective in therapy of pulmonary micrometastases.

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