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Original Article

Effects of Pulsing Procedure of Interleukin-12 in Combination with Interleukin-2 on the Activation of Peripheral Blood Lymphocytes Derived from Patients with Hepatocellular Carcinoma

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In patients with hepatocellular carcinoma (HCC), natural killer (NK) cell activity decreases significantly, and the reduced activity may be associated with the progression of HCC. In this study we evaluated the effects of pulsing with interleukin (IL)-2 and/or IL-12 on the activation of freshly isolated peripheral blood lymphocytes (PBL) derived from patients with HCC. PBL obtained from 9 HCC patients, 4 liver cirrhosis patients, and 9 normal subjects were cultured in the presence of IL-2 and/or IL-12. After 24 h of incubation, the levels of interferon (IFN)-γ and tumor necrosis factor (TNF)-α presented in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA). The IFN-γ and TNF-α production of PBL pulsed by a combination of IL-2 and IL-12 was significantly higher than those of PBL stimulated by either IL-2 or IL-12 alone. The mRNA encoding perforin, granzyme B, as well as IFN-γ and TNF-α, were markedly enhanced in PBL stimulated with a combination of IL-12 and IL-2. The pulsing procedure of IL-12 in combination with IL-2 resulted in the increase of IFN-γ and TNF-α, and the expression of perforin and granzyme B mRNA in PBL obtained from HCC patients, as well as in those obtained from normal subjects. These results indicate that adoptive immunotherapy based on PBL pulsed with a combination of IL-2 and IL-12 may be a promising adjunctive strategy for HCC treatment.

Key words: hepatocellular carcinoma (HCC), interleukin (IL)-2, interleukin (IL)-12, interferon (IFN)-γ, granzyme B

H epatocellular carcinoma (HCC) is responsible for significant morbidity and mortality in the Japanese adult population. The developments of several therapeutic modalities, such as resection, percutaneous ethanol injection therapy (PEIT) [1], and radio-frequency ablation (RFA) [2, 3], have enabled curative treatments of small HCC. For unresectable large HCC, lipiodole-chemoembolization and transcatheter arterial embolization have become the main treatment options [4–6]. However, HCC is liable to re-occur even after the curative treatment [7–10]. In addition, HCC is one of the malignant neoplasms showing poor response to anticancer agents, and the prognosis of patients with HCC remains poor.

In patients with HCC, the natural killer (NK) cell activity, which is believed to play an important role in

host anti-tumor defense mechanisms, appears to be significantly decreased, and the reduced activity was shown to be associated with the progression of HCC [11, 12]. Interleukin-12 (IL-12), a 75-kDa heterodimeric cytokine released by monocytes/macrophages early after immune stimulation, promotes the cytolytic maturation and proliferation of T, NK, and NKT cells and release of interferon (IFN)-γ from these effector cells [13–15]. Based on these findings, it may be possible to develop strategies to enhance the activation of peripheral blood mononuclear cells with IL-12, which may in turn provide a clue to developing an active immunotherapy for HCC.

The aim of this paper is to report our evaluation of the effects of pulsing with IL-12 and/or IL-2 on the activation of freshly isolated peripheral blood lymphocytes (PBL) derived from patients with unresectable large HCC. Our study was carried out to assess a novel therapeutic approach of adoptive immunotherapy for treating patients with unresectable large HCC.

Materials and Methods

Patients. Nine patients with unresectable large HCC (7 males and 2 females, median age 70) and four patients with liver cirrhosis (3 males and 1 female, median age 65), admitted to our hospital for diagnosis and treatment, were included in this study. Diagnosis of liver cirrhosis and HCC was based on characteristic clinical, laboratory, and serological features and findings of ultrasound sonography, abdominal computed tomography, and angiography. The clinical characteristics of the patients are shown in Table 1. Nine healthy volunteers (7 males and 2 females, median age 36) with no known liver disease served as control subjects. Informed consent was obtained from all patients and healthy control subjects.

Production of IFN-γ and TNF-α by IL-2- and/or IL-12-stimulated PBMC. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) centrifugation from the peripheral blood samples obtained from 9 patients with HCC, 4 patients with liver cirrhosis, and 9 healthy volunteers. The PBMC were washed three times in phosphate buffered saline (PBS) and suspended in 1×10^7 cells/ml in RPMI 1640 medium (GIBCO Laboratories Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU penicillin/ml, 100 mg streptomycin/ml, and 2 mMol of glutamin (culture medium).

The cells were plated in plastic plates (Becton Dickinson and Co., Cockeysville, MD, USA) and incubated for 2 h at 37 °C in a humidified CO_2 incubator. After the incubation, non-adherent cells were collected and used for the following experiments as peripheral blood lymphocytes (PBL).

PBL suspended in the culture medium were plated in 96-well microtiter plates (5×10^5 cells/100 μl/well) in the presence or absence of IL-2 and/or IL-12 (R&D Systems, Minneapolis, MN, USA) at indicated concentrations. After 24 h of incubation at 37 °C in 5% CO_2, the supernatants were collected and stored at -20 °C until the assays of TNF-α and IFN-γ. The levels of TNF-α and IFN-γ present in the supernatants were determined by commercially available kits (BioSource International, Inc., Camarillo, CA, USA).

RT-PCR for detecting IFN-γ, TNF-α, perforin, and granzyme B mRNA. Total RNA was prepared from PBL activated by the incubation with IL-2 and/or IL-12 for 24 h, and it was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) for expressing IFN-γ, TNF-α, perforin, and granzyme B mRNA. The PCR was carried out with complementary deoxyribonucleic acid (cDNA) derived from 100 ng RNA, 1 unit of Taq polymerase (Recombinant Taq DNA Polymerase; Takara Shuzo Co., Otsu, Japan), and reaction kits in a final volume of 20 μl. The PCR conditions included denaturation at 94 °C for 30 sec,

Table I Clinical profiles of patients with hepatocellular carcinoma and with liver cirrhosis

	HCC	LC
No. of cases	9	4
Gende (Male/Female)	7/2	3/1
Age (years)	70	65.5
HBV/HCV	1/8	1/3
Alb (3.9–4.9 mg/dl)	3.55 (2.73–3.89)	3.59 (3.06–3.96)
AST (11–32 IU/l)	76 (23–393)	30 (24–152)
ALT (6–39 IU/l)	33 (15–288)	18 (15–201)
AFP (< 7.0 IU/ml)	415 (7–85292)	7.15 (2.4–25.4)
PIVKA-II (< 28 mAU/ml)	16010 (225–248400)	14.5 (10–26)
ICG R15 (%)	33 (17.1–53.2)	25.5 (23–45.5)
Size of main tumor (cm)	7 (1.5–15)	–

∗, Values are expressed as Median (Minimum-Maximum). AFP, alpha-fetoprotein; ALT, alanine aminotransferase; Alb, albumin; AST, aspartate aminotrnsferase; HBV, hepatitits B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist-II.

annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. Thirty cycles of PCR were performed on each sample, after which the products were separated on 3% agarose gels.

The PCR primers used here for the amplification of IFN-γ, TNF-α, perforin, granzyme B, and β-actin-specific sequences were as follows; IFN-γ: 5'-GCATC GTTTTGGGTTCTCTTGGCTGTTACTGC-3' (sense strand), 5'-CTCCTTTTTCGCTTCCCTGTTTTAGCT GCTGG-3' (anti-sense strand) TNF-α: 5'-GAGTGAC AAGCCTGTAGCCCATGTTGTAGCA-3' (sense strand), 5'-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3' (anti-sense strand) perforin: 5'-GCAATGTGCATGTGTC TGTG-3' (sense strand), 5'-TTACCCAGGCTGAGTAC TGCT-3' (anti-sense strand) granzyme B: 5'-GGGGAAGC TCCATAAATGTCACCT-3' (sense strand), 5'-TACA CACAAGAAGGCCTCCAGAGT-3' (anti-sense strand) β-actin: 5'-ATCTGGCACCACACCTTCTACATTGA GCTGCG-3' (sense strand), 5'-CGTCATACTCCTGC TTGCTGATCCACATCTGC-3' (anti-sense strand). The PCR using these primers yield 427, 444, 572, and 838 bp, respectively.

Statistical analysis.   For multiple group comparisons, non-parametric comparisons were assessed by the repeated measure analysis of variance (ANOVA). When ANOVA indicated differences among the experimental groups, pairwise comparisons of each group were performed using the Scheffe test. A P value less than 0.05 was considered to be statistically significant.

Results

Production of IFN-γ and TNF-α from IL-2- and/or IL-12-stimulated PBL.   We evaluated the dose-dependent effects of IL-2 and/or IL-12 on the activation of freshly isolated PBL by monitoring the production of IFN-γ and TNF-α (Fig. 1A-F). PBL obtained from a patient with HCC and from a normal subject were incubated for 24 h with IL-2 alone at concentrations ranging from 0.05 to 500 U/ml (Fig. 1A and D), with IL-12 alone at concentrations ranging from 0.01 to 100 U/ml (Fig. 1B and E), or with 10 U/ml of IL-12 in combination with IL-2 at concentrations ranging from 0.05 to 500 U/ml (Fig. 1C and F). As shown in Fig. 1A, IFN-γ was not detected in the supernatants conditioned by non-activated PBL. The addition of exogenous IL-2 resulted in a slight increase in the IFN-γ production by PBL (Fig. 1A). On the other hand, low levels of

TNF-α were detected in the supernatants conditioned by non-activated PBL obtained from HCC patients and those from normal subjects (Fig. 1D). The addition of exogenous IL-2 used at various concentrations had no effect on the TNF-α production during the culture of PBL (Fig. 1D).

When PBL were stimulated with IL-12, the production of IFN-γ and TNF-α increased linearly with the dose of IL-12 in both PBL obtained from HCC patients and those obtained from normal subjects (Fig. 1B and E). In addition, as can be seen in Fig. 1C and F, the stimulation of PBL by 10 U/ml of IL-12 in combination with IL-2 at various concentrations resulted in dramatic increases in the production of IFN-γ and TNF-α in the conditioned supernatants. The effect of the combination of IL-2 and IL-12 on the production of IFN-γ and TNF-α was always more than that of IL-2 alone over a wide dose range. The same effect was observed on PBL obtained from HCC patients and from normal subjects.
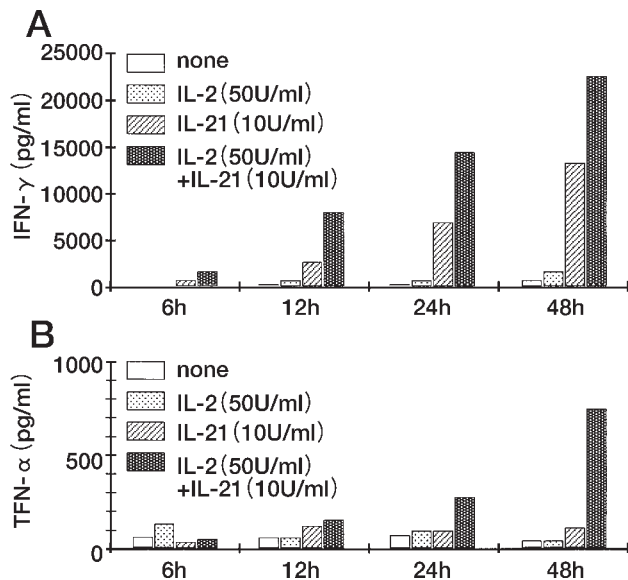
Next, we assessed the time-dependent effects of pulsing with IL-2 alone, IL-12 alone, and a combination of IL-2 and IL-12 on the production of IFN-γ and TNF-α by PBL. PBL (5×10^5 cells/100 μl/well) were cultured in the presence of IL-2 (50 U/ml), IL-12 (10 U/ml), or IL-2 (50 U/ml) and IL-12 (10 U/ml) for 6–48 h at 37 °C. At the times indicated, the cultured supernatants were harvested and assessed for the production of IFN-γ and TNF-α.

Data depicted in Fig. 2A show that the production of IFN-γ in the conditioned supernatants of PBL stimulated with IL-12 alone or with a combination of IL-2 and IL-12 increased in a time-dependent manner, whereas the level of IFN-γ in the supernatants of PBL stimulated with IL-2 alone did not increase during the culturing period. The production of TNF-α in the conditioned supernatants of PBL stimulated with a combination of IL-2 and IL-12 increased in a time-dependent manner. However, the level of TNF-α in the supernatants conditioned by either IL-2- or IL-12-stimulated PBL did not increase during the culturing period.

We examined the effect of IL-2 alone, IL-12 alone, and IL-12 in combination with IL-2 on the production of IFN-γ and TNF-α by PBL in 9 patients with HCC, 4 patients with liver cirrhosis, and 9 normal subjects (Fig. 3A and B). The analysis with repeated measure ANOVA revealed that there was no significant difference in the IFN-γ production between PBL obtained from normal subjects, LC patients, and HCC patients ($P = 0.692$)
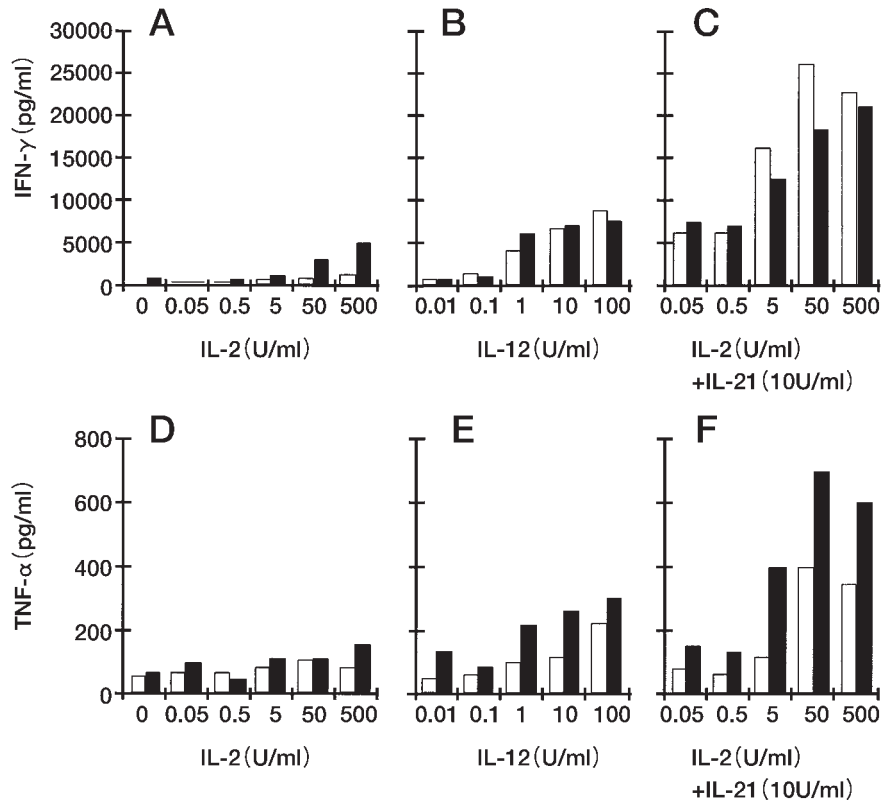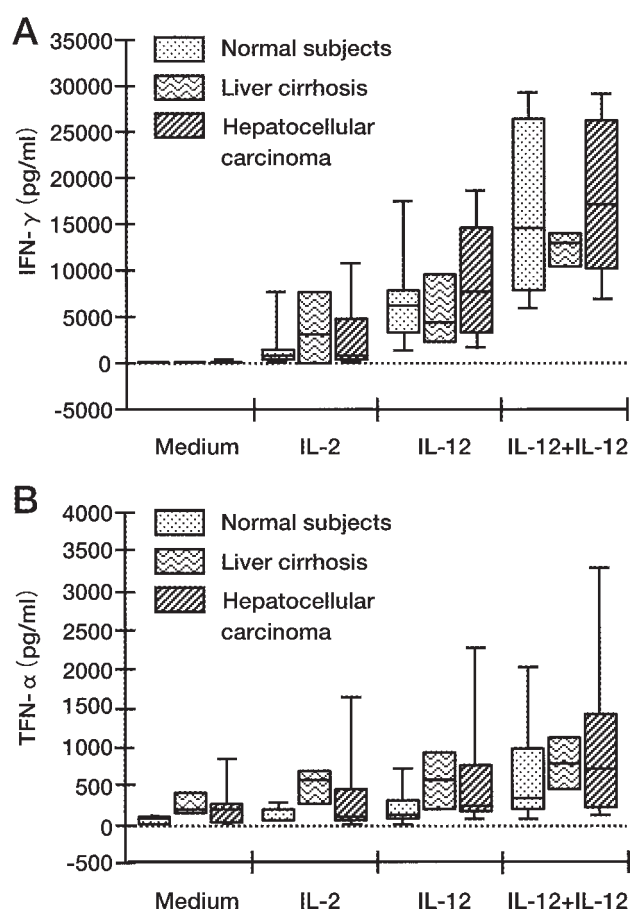
Fig. 1   Production of IFN-γ and TNF-α from IL-2- and/or IL-12-stimulated peripheral blood lymphocytes (PBL). PBL obtained from a normal subject (▢) and a patient with hepatocellular carcinoma (HCC) (■) were incubated for 24 h with IL-2 at concentrations ranging from 0.05 to 500 U/ml (**A** and **D**), or with IL-12 at concentrations ranging from 0.01 to 100 U/ml (**B** and **E**), or 10 U/ml of IL-12 in combination with IL-2 at concentrations ranging from 0.05 to 500 U/ml (**C** and **F**). After incubation, the levels of IFN-γ (**A**, **B**, and **C**) and TNF-α (**D**, **E**, and **F**) present in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA).

Fig. 2   Time-dependent effect of pulsing with IL-2 alone, IL-12 alone, and a combination of IL-12 and IL-2 on the production of IFN-γ (**A**) and TNF-α (**B**) by peripheral blood lymphocytes (PBL). PBL (5×10^5 cells/100 μl/well) were cultured in the absence (▢) or presence of IL-2 (50 U/ml) (▨), IL-12 (10 U/ml) (▨), or IL-2 (50 U/ml) and IL-12 (10 U/ml) (▨) for 6, 12, 24, and 48 h. At the time indicated, the cultured supernatants were harvested and assessed for the production of IFN-γ and TNF-α.

(Fig. 3A). The analysis showed that there were significant differences in IFN-γ production between PBL stimulated by either IL-2 or IL-12, and those stimulated with a combination of IL-2 and IL-12 ($P < 0.0001$ by the repeated measure ANOVA) (Fig. 3A). The IFN-γ production of PBL stimulated by IL-12 (normal subjects, median = 6306 pg/ml; LC, 4300 pg/ml; HCC, 7632 pg/ml) was higher than that of PBL stimulated by IL-2 (normal, 751 pg/ml; LC, 3121 pg/ml; HCC, 822 pg/

ml) ($P < 0.005$ by Scheffe test). In addition, the IFN-γ production of PBL pulsed by the combination of IL-2 and IL-12 (normal subjects, median = 14500 pg/ml; LC, 12978 pg/ml; HCC, 17140 pg/ml) was significantly higher than that of PBL stimulated by either IL-2 or IL-12 ($P < 0.0001$ by Scheffe test).

In addition, we found a significant difference in TNF-α production among PBL stimulated either by IL-2, IL-12, or the combination of IL-2 and IL-12 ($P < 0.0001$ by repeated measure ANOVA). The TNF-α productions of PBL stimulated by the combination of IL-2 and IL-12 (normal subjects, median = 340 pg/ml; LC, 784 pg/ml; HCC, 710 pg/ml) were significantly higher than those of PBL stimulated by IL-2 (normal subjects, 70 pg/ml; LC, 573 pg/ml; HCC, 115 pg/ml) ($P = 0.0002$ by Scheffe test) and those of PBL stimulated by IL-12 alone (normal subjects, 130 pg/ml; LC, 586 pg/ml; HCC, 255 pg/ml) ($P = 0.012$ by Scheffe test) (Fig. 3B). The analysis confirmed that PBL derived from HCC patients could be activated as well as PBL derived from normal subjects by pulsing with the combination of IL-2 and IL-12.

Expression of perforin, granzyme B, TNF-α, and IFN-γ mRNA. The studies further examine whether the expression of perforin and granzyme B, which are proteins involved in relevant cell-mediated cytotoxicity, were enhanced in IL-2- and/or IL-12-stimulated PBL obtained from normal subjects and HCC patients (Fig. 4). The expression of perforin and TNF-α mRNA were readily detected in unstimulated PBL obtained from a HCC patient and those from a normal subject. The granzyme B mRNA was weakly expressed in PBL stimulated with 50 U/ml of IL-2, whereas no expression of IFN-γ mRNA was observed after stimulation with IL-2 alone. In PBL stimulated with 50 U/ml of IL-12, the expression of granzyme B and IFN-γ mRNA were up-regulated, as well as the expression of perforin and TNF-α mRNA. Furthermore, as can be seen in Fig. 4, the mRNA encoding perforin, granzyme B, TNF-α, and IFM-γ were markedly and significantly enhanced in PBL stimulated with the combination of IL-2 and IL-12. There were no significant differences in the expression of granzyme B mRNA and IFN-γ mRNA between PBL obtained from normal subjects and those from HCC patients.

Fig. 3   The production of IFN-γ (A) and TNF-α (B) by peripheral blood lymphocytes (PBL) obtained from 9 normal subjects (▦), 4 patients with liver cirrhosis (▧), and 9 patients with hepatocellular carcinoma (HCC) (▨). Peripheral blood lymphocytes (5×10^5 cells/100 μl/well) were cultured in the absence (Medium) or presence of IL-2 (50 U/ml) (IL-2), IL-12 (10 U/ml) (IL-12), or IL-2 (50 U/ml) and IL-12 (10 U/ml) (IL-2 + IL-12) for 24 h. After the incubation, the levels of IFN-γ and TNF-α present in the supernatants were determined by ELISA. Medians are indicated with horizontal bars. The vertical bars indicate the range, and the horizontal boundaries of the boxes represent the first and third quartiles.

Discussion

The development of new therapies such as immunotherapy is essential to prevent the recurrence of HCC. Recently, Takayama *et al.* reported that adoptive immunotherapy using autologous lymphocytes activated in vitro with recombinant IL-2 and anti-CD3 antibody decreased the frequency of the recurrence of HCC and improved recurrence-free outcomes following HCC surgery by 18% compared with controls [16]. However, the overall survival did not differ significantly between the immunotherapy group and the control group.

On the other hand, it was reported that the NK cell activity was significantly decreased in HCC patients compared with that in the control subjects, which suggested that preoperative NK cell activity will help predict recurrence and prognosis after hepatectomy in patients with HCC [11]. Also, Kawarabayashi *et al.* reported that the proportion of CD56 + NKT cells and NK cells decreased in liver mononuclear cells derived from cirrhotic livers, which suggested that a decreased number of CD56 + NKT cells and NK cells in cirrhotic livers may be related to their susceptibility to HCC [12]. From these findings, we conclude that adoptive immunotherapy based on activated NK cells and NKT cells may be a promising adjunctive strategy for avoiding the recurrence of hepatocellular carcinoma after curative treatment.

IL-12 is a heterodimeric cytokine that has been shown to cause the proliferation of activated T cells and NK cells, to enhance the lytic activity of NK cells, and to induce IFN-γ production by resting and activated T and NK cells [13–15, 17, 18]. In mouse models of HCC, IL-12 gene therapy has been reported to elicit protective and therapeutic immunity against hepatocellular carcinoma. Intratumoral administration of mouse IL-12 vector elevated serum IL-12 and IFN-γ, induced more lymphocyte infiltration by NK cells and CD3 + cells into the tumor, and reduced the number of microvessels [19, 20].

In this study, elevated IFN-γ levels were observed in the supernatants of PBL stimulated by IL-12 for 24 h compared with those stimulated by IL-2. In addition, IL-12 pulsing was found to up-regulate the expression of their cytolytic component, granzyme B mRNA, compared with IL-2 pulsing. These observations suggest that pulsing with IL-12 can induce IFN-γ production by T and NK cells and enhance the lytic activity of NK cells even in PBL obtained from patients with HCC.

Pulsing with a combination of IL-2 and IL-12 augmented IFN-γ and TNF-α production by PBL in a synergistic or cumulative manner. Also, the mRNA encoding perforin and granzyme B were markedly and significantly enhanced in PBL pulsed with the combination of IL-2 and IL-12 for 24h in comparison with those pulsed with either IL-2 alone or IL-12 alone. The pulsing procedure of IL-12 in combination with IL-2 for 24 h resulted in increased IFN-γ and TNF-α production and increased expression of perforin and granzyme B mRNA
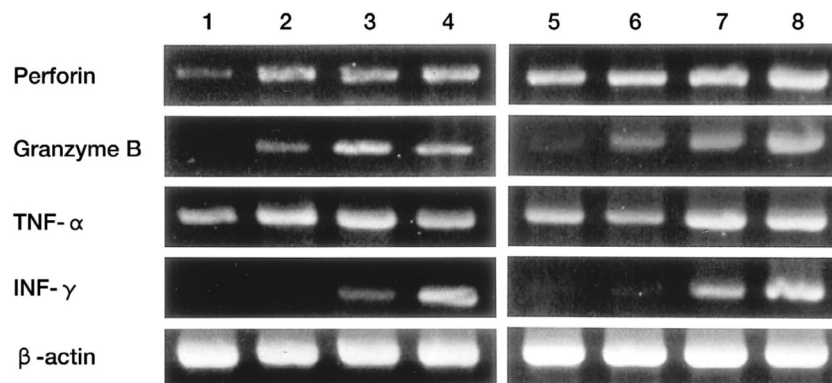
Fig. 4   Expression of perforin, granzyme B, TNF-α, and IFN-γ mRNA in peripheral blood lymphocytes (PBL) stimulated by IL-2 and/or IL-12. PBL obtained from a normal subject (1, 2, 3 and 4) and from a patient with hepatocellular carcinoma (HCC) (5, 6, 7 and 8) were cultured in the absence (1 and 5) or presence of IL-2 (50 U/ml) (2 and 6), IL-12 (10 U/ml) (3 and 7), or IL-12 (50 U/ml) plus IL-12 (10 U/ml) (4 and 8) for 24 h. Total RNA was prepared from activated PBL and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) for the expression of perforin, granzyme B, TNF-α, and IFN-γ mRNA.

in PBL obtained from HCC patients as well as in those obtained from normal subjects.

Chan et al. [21] reported that IL-12 synergizes with IL-2 in the accumulation of IFN-γ mRNA in PHA-activated T cell blasts and the T leukemia cell line TALL-103/2. Synergy between IL-2 and IL-12 can be demonstrated only at the level of mRNA stability, and both cytokines are required to increase IFN-γ mRNA half-life. Also, in the human NK cell line NK3.3, IL-12 and IL-2 synergized in the induction of IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) and their synergy was attributed to an increased accumulation and stability of the IFN-γ and GM-CSF mRNA [22]. In addition, it was reported that co-incubation with IL-12 and IL-2 augmented IFN-γ and TNF-α production by non-adherent mononuclear cells in a synergistic or cumulative manner, and also that the NK cell cytotoxicity was much more augmented by the co-incubation of IL-12 and IL-2 than the incubation of IL-12 alone [23].

Activated lymphocytes including NK cells might be essential for an anti-tumor effect *in vivo*. Adoptive immunotherapy with enhanced natural cytotoxicity mediated NK cells might play an important role in host anticancer defense mechanisms *in vivo*. PBL pulsed with a combination of IL-2 and IL-12 could be used as effector cells in adoptive immunotherapy, although the specificity of cytotoxic effects of these activated PBL against HCC should be evaluated. In the treatment of HCC, a relative sufficient number of activated lymphocytes can be administered to a liver containing HCC with reasonable safety by transcatheter injection. Also, activated lymphocytes can be continuously administered to the liver through a catheter placed into the hepatic artery. Therefore, adoptive immunotherapy based on PBL pulsed with a combination of IL-2 and IL-12 may be a promising adjunctive strategy for HCC treatment.

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