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理事長 殿

研究者氏名 蘇 偉 (蘇) ㊞
所属機関名 大阪大学 臓器制御外科学(第一外科)
指導責任者氏名 伊藤 壽記
職名 助教授
所在地 〒565-0871 吹田市山田丘2-2 E1
電話 06-6879-3153 内線 3153

1．研究テーマ　大腸癌肝転移の制御に関する実験的研究

2．本年度の研究業績

(1) 学会・研究会等における発表　(有)・無　(学会名・演題)

Su W., Ito T., Kitagawa T., Oyama T.,et al: Immuno-gene therapy using IL-12 transduced fibroblasts in murine liver metastasis models. 第７回日本遺伝子治療学会総会，東京，２００１年７月７日。

Su W., Ito T., Kitagawa T., Oyama T.,et al: Immuno-gene therapy using IL-12 transduced fibroblasts in murine liver metastasis models. 第５６回日本消化器外科学会総会，秋田，２００１年７月２６日。

(2) 学会誌等に発表した論文　有・(無)　(雑誌名・論文名)

3．今後の研究計画

私は今年3月に大阪大学大学院医学研究科臓器制御外科学（第一外科）博士課程を卒業し、博士学位を取得し、5月に中国北京医院普通外科に帰るつもりです。北京に帰りましたら、日本で研究した大腸癌肝転移の免疫治療、遺伝子治療を続けてやりたいと思います。中国でこの研究に関する動物実験を立ち上げます、臨床応用の可能性を検討します。

4．指導責任者の意見

蘇偉君は、平成10年4月 大阪大学大学院医学研究科（臓器制御外科学）に入学し、消化器腫瘍に対する遺伝子免疫療法についての研究に従事してきた。研究態度はまじめで4年間で3編の英文論文（1編はpublished，2編は投稿中）を作成しました。研究内容は、消化器癌の肝転移の制御に向けて、自己腫瘍細胞に強力な抗腫瘍サイトカインレセプターを遺伝子導入して、門脈経由で投与するというもので、臨床応用に向け実現性の高いものと評価しています。

指導責任者氏名 千葉喜記 ㊞

5．研究報告書

別紙報告書作成要領により、添付の用紙で研究報告書を作成して下さい。

研究発表中または研究中の本人のスナップ写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

※発表に当っては、*日中医学協会助成金による*旨を明記して下さい。

—日中医学協会助成事業—

The Anti-tumor Effects of Intra-portal Injection of Fibroblasts Genetically Engineered to Secrete IL-12 in Murine Liver Metastasis Models

研究者氏名　蘇　偉
中国所属機関　北京医院普通外科
日本研究機関　大阪大学臓器制御外科学（第一外科）
指導責任者　助教授　伊藤壽記
共同研究者　北川　透、大山　司、李　千万

Abstract

Augmentation of the hepatic immune system with IL-12 administration can prevent and treat liver metastasis. Fibroblasts genetically engineered to secrete IL-12 have been shown to possess anti-tumor activity in the subcutaneous tumor models. In this study, the anti-tumor effect of intra-portal injection of fibroblasts genetically engineered to secrete IL-12 was investigated in murine liver metastasis models. Transfected fibroblasts (3T3-IL-12, 3T3-neo) were obtained from NIH-3T3 cells transfected with retroviral vectors. Intra-portal administration of 3T3-IL-12 cells on day 0 suppressed the tumor establishment in MC-38 liver metastasis models. In cell tracking experiment, the red fluorescent cells were mainly detected in the liver after intra-portal injection of PKH-26-labeled 3T3-IL-12 cells, but few in other organs. And these cells remained in the liver even after 7 days. IL-12 and IFN-γ levels in the serum and in the liver extracts were up-regulated by the intra-portal injection of 3T3-IL-12 cells. Intra-portal administration of 3T3-IL-12 cells suppressed the establishment of liver metastasis in a dose-dependent manner. Intra-portal administration of 3T3-IL-12 cells on day 5 suppressed the growth of established tumors in MC-38 liver metastasis model. The intra-portal injection of 3T3-IL-12 cells induced lymphocytic infiltration in MC-38 tumors. After the treatment with 3T3-IL-12 cells, IFN-γ production by splenocytes re-stimulated with irradiated parental tumor cells *in vitro* was significantly increased. Specific cytotoxicities of splenocytes against parental tumor cells *in vitro* also were induced by intra-portal injection of 3T3-IL-12 cells. These findings suggested that intra-portal injection of IL-12-producing fibroblasts would be an effective strategy for the treatment of liver metastasis.

Key Words　IL-12, fibroblast, liver metastasis, gene therapy

Introduction

IL-12, a disulfide-linked heterodimer consisting of 40-kDa and 35-kDa subunits, is originally produced by macrophages, dendritic cells and granulocytes. IL-12 presents marked therapeutic effects in a wide variety of tumor models[1–7], involving NK cells, T cells, macrophages, dendritic cells and tumor cells. IL-12 stimulates NK cells to produce IFN-γ, induces Th1 development, enhances CTL to kill tumor cells, activates macrophages, modulates activity of dendritic cells and inhibits angiogenesis[2–7]. However, systemic administration of IL-12 has caused dose-dependent and schedule-related toxicity in clinical trails. In the first clinical trial, systemic therapy of IL-12 resulted in the death of 2 patients and led to severe toxic effects in other 15 patients[8].

Tahara *et al* started a clinical trial for melanoma, head and neck cancer, and breast cancer by weekly injections of IL-12-producing fibroblasts at the site of palpable tumors[9]. We thought that intro-portal administration of IL-12-transduced fibroblasts should be effective to induce production and localization of IL-12 in the liver. Though there are several reports about systemic administration of IL-12 for liver metastasis in murine models[10, 11], to our knowledge, there are few reports about intro-portal administration of IL-12-transduced cells for liver metastasis. In this study, we investigated the anti-tumor effect of intro-portal administration of IL-12-producing fibroblasts to prevent and treat the liver

metastasis in the murine models.

Material and methods

Cell lines and mice MC-38, a poorly immunogenic C57BL/6 murine colon adenocarcinoma cell line, was kindly provided by Dr. Steven A. Rosenberg (Nation Cancer Institute, USA). Female 6- to 10-weeks-old C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). Mice were fed in the Institute of Experimental Animal Sciences of Osaka University Graduate School of Medical, and routinely screened and found to be specific pathogen free.

***In vivo* tumor experiments** Under the anesthesia of ketamine hydrochloride (Sankyo Co., Tokyo, Japan), C57BL/6 mice underwent laparotomy. In the liver metastasis models, tumor cells were injected into the portal vein on day 0. After the mice were sacrificed on day 21, the liver was weighed and cut to slices each 3 mm thick. The number of metastatic nodules was counted using a dissecting microscope. In intracutaneous tumors, mice were shaved over the right flank and were injected intracutaneously with a tumorigenic dose of tumor cells. The growth of the intracutaneous tumors was monitored in the next 21 days, and the tumor sizes (square millimeters) were shown as the products of the perpendicular diameters of each tumor.

***In vivo* cell tracking experiment** PKH-26 (Sigma Chemical Co, USA) is a red fluorescent cell linker, which was used *in vitro* and *in vivo* cell tracking application[12]. 3T3-IL-12 cells were labeled with PKH-26 as described previously[12]. PKH-26-labeled 3T3-IL-12 cells were injected into the portal vein. After 2hr, 1, 2, 3, 5, or 7 days, the mice were sacrificed and harvested organs were embedded in OCT compound (Sakura Funetechnical Co., Ltd., Tokyo, Japan), and stored at –80°C stored. PKH-26-labeled cells were observed through a fluorescent microscope.

Statistical analysis Statistical analysis of experiments was performed using Student's t-test and ANOVA test when involved growth of intracutaneous tumors. Data are presented as mean±SD. P values less than 0.05 were considered as statistically significant.

Results

IL-12 production by genetically engineered fibroblasts *in vitro*. To determine IL-12 production by 3T3-IL-12 cells, $1x10^6$ transfected fibroblasts were cultured in a 75-cm^2 culture dishes for 48 hr, then supernatants were collected. IL-12 production by 3T3-IL-12 cells was 45.5±6.8 ng/10^6 cells/24 hr. IL-12 production by 3T3-neo cells or non-transfected MC-38 cells was not detected, <0.05 ng/10^6 cells/24 hr.

Intra-portal administration of 3T3-IL-12 cells on day 0 suppressed the tumor establishment in MC-38 liver metastasis models. After intra-portal injection of MC-38 cells, $1x10^5$ of 3T3-IL-12 cells were injected intra-portally or intraperitoneally on day 0, and the same amount of 3T3-neo cells or HBSS were injected into the portal vein as controls at same time. The livers were removed on day 21, and the liver weight and the number of metastatic nodules were examined. The liver weight of the mice treated with 3T3-IL-12 intraperitoneally significantly less than those of mice treated with 3T3-neo cells (P=0.0441), and the liver weight of the mice treated with 3T3-IL-12 intra-portally also significantly less than those of mice treated with 3T3-neo cells (P=0.0006). The numbers of metastatic nodules in the 3T3-IL-12 intraperitoneal treatment group or in the 3T3-IL-12 intra-portal treatment group were significantly less than that in the 3T3-neo group (P<0.05).

Intra-portally injected 3T3-IL-12 cells remained in the liver, and up-regulated IL-12 and IFN-γ levels in the sera and the liver extracts. To trace intra-portally injected 3T3-IL-12 cells, $1x10^5$ of PKH-26-labeled 3T3-IL-12 cells were injected into the portal vein, while unlabeled 3T3-IL-12 cells were used as controls. The red fluorescent cells were observed in the liver 2hr after the injection, but few in the lung, the brain, the kidney, the spleen and the muscle. Further, these cells were continually observed in the liver in the next 7 days, but not in the other organs at all.

Sera and liver extracts were collected after the intra-portal injection of 3T3-IL-12 cells from day 1 to day 14. The IL-12 levels in the serum or in the liver extracts peaked 5 days after intra-portal injection of 3T3-IL-12 cells and

gradually decreased until 14 days. The peak of IFN-γ levels in the serum or in the liver extracts occurred 7 days after the injection, delayed a few days compared with those of IL-12. Elevations of IL-12 or IFN-γ levels were not observed in the mice injected with 3T3-neo cells into the portal vein.

Intra-portal administration of 3T3-IL-12 cells suppressed the establishment of MC-38 liver metastasis in a dose-dependent manner. After intra-portal injection of MC-38 cells, $3x10^3$, $1x10^4$, $3x10^4$ or $1x10^5$ of 3-IL-12 cells were injected into the portal vein on day 0. The significant decrease was observed at the dose of $1x10^4$ 3T3-IL-12 cells/mouse, compared with those of the 3T3-neo group ($P<0.05$). The strongest anti-tumor effect was obtained in the groups using $1x10^5$ 3T3-IL-12 cells/mouse. Intra-portal injection of 3T3-IL-12 cells suppressed the establishment of liver metastasis in a dose-dependent manner.

Intra-portal administration of 3T3-IL-12 cells on day 5 suppressed the growth of established tumors in MC-38 liver metastasis models. The established liver metastasis of MC-38 was treated with 3T3-IL-12 cells. After 5 days of intra-portal inoculation of MC-38 cells, $1x10^5$ of 3T3-IL-12 cells were injected into the portal vein. After 21 days, the liver weight and the number of metastatic nodules examined. The liver weight and the number of metastatic nodules in the 3T3-IL-12-treatment group significantly less than those in the 3T3-neo group ($P<0.05$). Intra-portal injection of 3T3-IL-12 cells on day 5 suppressed the growth of established tumors in MC-38 liver metastasis models.

Histological expression of liver with MC-38 metastasis showed that lymphocytes slightly infiltrated in the MC-38 tumors of the HBSS group or the 3T3-neo group. There were, however, lymphocytes markedly infiltrated in the MC-38 tumor of the 3T3-IL-12-treatment group. The intra-portal injection of 3T3-IL-12 cells induced lymphocytic infiltration in MC-38 tumors.

IFN-γ production by splenocytes re-stimulated with irradiated parental tumor cells was increased after the treatment with 3T3-IL-12 cells. Splenocytes harvested from mice with liver metastasis of MC-38 in each treatment group were re-stimulated *in vitro* for 48 hr with 20 Gy irradiated parental tumor cells or YAC-1 cells to evaluate their ability to produce IFN-γ and IL-4. IFN-γ production by splenocytes from mice with liver metastasis of MC-38 was significantly increased in the 3T3-IL-12-treatment group as compared with in the 3T3-neo group ($P<0.05$). After these splenocytes were re-stimulated with irradiated YAC-1 cells that were used as non-specific controls, IFN-γ production by these splenocytes was not significantly increased. No significant difference of IL-4 production was observed among these treatment groups. These results suggest that the treatment of 3T3-IL-12 cells induced a Th1 immune response specific to the parental tumor cells.

Specific cytotoxicities against parental tumor cells were induced by intra-portal injection of 3T3-IL-12 cells. Splenocytes were harvested from mice with liver metastasis on 14 days after implantation of tumor cells in each treatment group. Splenocytes from mice with liver metastasis of MC-38 presented a trend of increased cytotoxicities against parental cells after the treatment of 3T3-IL-12 cells, and a significant difference was observed between 3T3-IL-12 cells and 3T3-neo cells treatment groups ($P<0.05$). Cytotoxicities against MC-38 cells were higher than that against YAC-1 cells that were used as non-specific controls. There was a significant difference between cytotoxicities against MC-38 cells and against YAC-1 cells. Similar results were obtained using liver mononuclear cells derived from mice with liver metastasis. These results suggest that the treatment of 3T3-IL-12 cells induced specific cytotoxicities to the parental tumor cells.

Discussion

According to the previous observations in animal models, it is suggest that IL-12 is a promising candidate reagent using in the tumor immunotherapy[2—7, 13--15]. In our study, micro-foci of liver metastases were detected histologically and grossly on day 3 after intra-portal injection of MC-38 cells. We injected rIL-12 from day 3 to confirm that intraperitoneal or intrasplenic injection of rIL-12 was efficient on suppress MC-38 tumor growth of liver metastasis. Moreover, intraperitoneal or intra-portal administration of 3T3-IL-12 cells on day 0 also caused an anti-tumor effect to

suppress liver metastasis of MC-38. Though there was not a significant difference between the anti-tumor effects of intraperitoneal administration and intra-portal administration of 3T3-IL-12 cells, intra-portal administration of 3T3-IL-12 cells seemed to be better.

After intra-portal injection of 3T3-IL-12 cells, 3T3-IL-12 cells were observed in the liver, but few in other organs. These cells remained in the liver in the next 7 days. Further, intra-portal injection of 3T3-IL-12 cells maintained the higher levels of IL-12 and IFN-γ in the serum and the liver extract at least during two weeks. These results suggested intra-portal injection of 3T3-IL-12 cells induced a selective localization of 3T3-IL-12 cells and these cells produced IL-12 in the liver.

The anti-tumor effect of intra-portal injection of 3T3-IL-12 cells was confirmed in several liver metastasis models. At first, intra-portal injection of 3T3-IL-12 cells on day 0 suppressed the establishment of MC-38 liver metastasis. In B16-BL6 (murine melanoma) or Panc-02 (murine pancreas adenocarcinoma) liver metastasis models, intra-portal injection of 3T3-IL-12 cells on day 0 also suppressed the tumor establishment (data not shown). Then we showed that intra-portal administration of 3T3-IL-12 cells suppressed the establishment of MC-38 liver metastasis in a dose-dependent manner. It was suggested that the amounts of IL-12 produced in the liver was important for this anti-tumor effect. Moreover, after 5 days of intra-portal injection of tumor cells, when the micro-foci of liver metastases had formed, intra-portal administration of 3T3-IL-12 cells was also effective in the established liver metastasis of MC-38. Intra-portal administration of 3T3-IL-12 not only suppressed the establishment of liver metastasis, but also suppressed the growth of established micro-foci of liver metastases.

Localization of IL-12 production at the tumor site could have an advantage over systemic treatments by inducing an immune response against regressing tumor without the need for circulating high levels of IL-12[16]. Regional application of IL-12 has been examined in subcutaneous tumor models using the peritumoral injection of fibroblasts engineered to secrete IL-12 to activate a local anti-tumor immune response which also promoted systemic immune protection specific to the tumor cells[14, 15]. It was reported that IL-12 directly effect on tumor cells[17, 18] and the amount of IL-12 made available at the tumor site is critical for tumor regression[19], so this strategy appears to have greater advantage over systemic administration for liver metastasis. Systemic administration of IL-12 has caused dose-dependent and schedule-related toxicity in the clinical trails[8, 20]. The local administration of IL-12 producing cells at tumor site is believed to be effective in minimizing side effects, because there is with lower systemic concentration of IL-12[15, 21]. Recently, a phase I clinical trial showed that peritumoral injection of IL-12-transduced fibroblasts was feasible in patients with advanced breast cancer[22]. Clinically significant toxicities were not encountered, and transient but clear reductions of tumor sizes were observed in four of nine cases[22].

As a summary, intra-portal injection of IL-12-producing fibroblasts into liver may be an effective strategy for the treatment of liver metastasis.

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注：本研究は、２００１年７月２６日「第５６回日本消化器外科学会総会」にて発表いたしました。