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Investigation of the Anti-Tumor activity of Hepatic Kupffer Cells for Control of Colon Cancer Micro-Metastasis to the Liver via the Fas/Fas ligand system

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Summary

Kupffer cells are macrophages present in the sinusoids of the liver, and they contribute to immunity by phagocytosis of wastes in the body, killing of pathogens, and secretion of various cytokines. Recently it was reported that Kupffer cells contribute to the immune mechanism in that they express Fas ligand and induce apoptosis in Fas bearing cytotoxic T cells. In this study,we investigated whether or not Kupffer cells induce apoptosis in cancer cells through the Fas/Fas ligand pathway.

Fas ligand expression was observed in Kupffer cells. A significant increase was seen in apoptosis of cancer cells co-cultured with Kupffer cells. Furthermore, the increased apoptosis of cancer cells was inhibited by anti-Fas ligand monoclounal antibody. These results suggest that Kupffer cells can induce apoptosis in cancer cells via the Fas/Fas ligand system.

Introduction

Recent technical improvements in surgery have improved therapeutic results in colon cancer. Nevertheless, even in cases of radical surgery, numerous instances of localized recurrence or metastasis to the liver have been reported[1-3]. Regarding localized recurrence, potential solutions include achievement of an adequate incision line and prevention of implantation during surgical manipulation. For the prevention of liver metastasis, control of micro-metastasis is paramount. Cancer cells generated locally enter the blood stream, reach the hepatic sinusoids via the portal vein system, adhere to vascular endothelial cells and invade the sinusoids[4], where there are Kupffer cells and hepatic lymphocytes that act to prevent cancer cell intrusion[5,6]. It has been reported that hepatic lymphocytes exhibit higher anti-tumor effect than lymphocytes in peripheral blood[7]. On the other hand, hepatic Kupffer cells are macrophages that dwell in the sinusoids of the liver. They possess a broad range of functions for biophylaxis[8,9]. It is known that one of these functions is to discharge NO or super oxidant, thus exhibiting an anti-tumor effect against cancer cells[10-13]. Recently, it has been reported that Kupffer cells express Fas ligand and induce apoptosis in Fas bearing cytotoxic T lymphocyctes (CTL)[14,15]. Accordingly, if cancer cells that have entered via the portal vein blood flow express Fas, it is suspected that, aside from NO or reactive oxygen species, there is an attack by Kupffer cells via the Fas-Fas ligand system. It has been confirmed that cancer cells express Fas at local sites[16,17] in colon cancer.

In the present study, we investigated whether or not hepatic Kupffer cells exhibit an anti - tumor effect

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against cancer cell strains via the Fas-Fas ligand system.

Materials and Methods

1. Animals

Male Lewis rats weighing 250 g to 300 g were used in experiments. The animal experiments were conducted in accordance with the Tokyo Medical University guidelines on animal experiments, based on standards governing animal protection and storage (Ordinance No. 105) and on standards governing experimental animals and their storage (Prime Minister's Office Notification No. 6).

2. Cancer cell strains

The cancer cell strain used was colon 26, a colon cancer cell strain that causes liver metastasis originating in BALB/c mice, it was cultured in RPMI 1640 medium to which was added 10% FBS, 5 U/ml of penicillin and 5 μg/ml of streptomycin. Used as positive control was the Jurkat cell, a Fas-expressing cell strain that originates from human T lymphoma, it was cultured in the same medium as colon 26.

3. Isolation of Kupffer cells

Kupffer cells were isolated from rats according to the procedure described by Smedsrod, Braet et al[18]. Briefly, the liver was perfused with 100 ml of Ca^{2+}-free Hanks balanced salt solution (HBSS) (GIBCO BRL, Rockeville, MD, USA), a pre-perfusion solution, at 37°C at the rate of 20 ml/min. Then, 100 ml of Ca^{2+}-rich HBSS containing 0.05% Type IV collagenase was used for perfusion. Following excision, the liver was mashed in digestion fluid (DMEM/F-12, 100 ml, 0.02% Pronase E, 0.025% Type IV collagenase + 10 mg/ml DNase 10 mM HEPES, 5 U/ml penicillin, 5 μg/ml streptomycin). This was allowed to oscillate in warm water at 37°C for 20 min, then filtered through gauze. The suspension was centrifuged at 300 g, 4°C for 5 min. The pellet was washed in washing buffer (Ca^{2+}-rich HEPES). Washing buffer was again added to the pellet, the suspension was centrifuged at 4°C for 1 min again. Hepatic parenchyma was allowed to precipitate, and was then removed. Then, the supernatant was centrifuged at 300 g, 4°C for 5 min, the pellet was suspended in Ca^{2+}-rich HEPES, and this was stratified in 50% Percoll and 30% Percoll fluid. Following centrifugation at 3,000 g, 4°C for 20 min, the second layer was collected, washed in washing buffer, then suspended in 10% FBS and RPMI 1,640 with 5 U/ml of penicillin and 5 μg/ml of streptomycin. It was cultured for 10 min at 37°C in a 5% CO_2 incubator with FBS coating flask. Thereafter, the flask was tapped and the culture medium was discarded. The adhering cells were Kupffer cells. Phagocytosis of 100 μm beads was performed for these cells, and it was confirmed that Kupffer cells were at 95% or more. (Most of the other 5% cells were endothelial cells.)

4. Confirmation of Kupffer cell Fas ligand expression

For expression at the mRNA level, reverse transcription polymerase chain reaction (RT-PCR) was done in accordance with the method of Muschen et al[15]. The forward 5′-ATGGAACTGCTTTGATCTCTGG and reverse 3′-AGATTCCTCAAAATTGATCAAGA. were used for RT-PCR. At the protein level, anti-rat Fas ligand monoclonal antibody (FasL mAb) (clone 33, BD Transduction Laboratories, Franklin Lake , NJ, USA) was held in the primary antibody, and Western blotting was performed.

5. Induction of apoptosis in cancer cells by Kupffer cells

On a chamber slide, 100 μl culture supernatant of Kupffer cell (5×10^6 cells/ml) was added to colon 26 cells (10^5 cells/ml) and cultured for 4 hrs. After washing, an apoptosis detection kit, annexinV-Cy3 (Sigma, Saint Louis, MO, USA) was used to label cells, which were then observed in a 450 nm fluorescence microscope. In the present study, we had inhibition experiment by adding 20 ng/ml anti FasL mAb when Kupffer cells were mixed with target cells and culturing 4 hrs. At this juncture we used goat IgG as an isotype of anti FasL mAb.

6. Quantitative apoptosis assay

For assaying the apoptosis-inducing potential of Kupffer cells, a DNA fragmentation kit was used. Firstly, colon 26 (2×10^5 cells) were cultured over one night in 10 μM BrdU and labeled. Next, labeled colon 26 and Kupffer cells (2×10^6 cells) were mixed and cultured for 4 hrs on a 96-well microplate. Upon completion of mixed cultivation, 250 g was centrifuged for 10 min and the supernatant was removed. After the formation of lysate in lysis buffer, the suspension was transferred to an anti-DNA coating plate in increments of 100 μl/well. Following DNA cleavage by microwave, peroxidase labeled - anti BrdU antibody was used, and labeled DNA fragments were measured by ELISA. We also performed an inhibition experiment as soon as experiment 5 (Induction of apoptosis in cancer cells by Kupffer cells)

Results

1. Kupffer cell Fas ligand expression

Upon investigation of Fas ligand expression by rat Kupffer cells, Fas ligand expression on the mRNA and protein levels was confirmed by RT-PCR and Western blotting (Fig. 1).

2. Induction of apoptosis in cancer cells by Kupffer cells

We investigated whether Kupffer cell Fas ligand induces apoptosis in colon 26 cells. Kupffer cell supernatant was added to colon 26, labeled with annexinV-

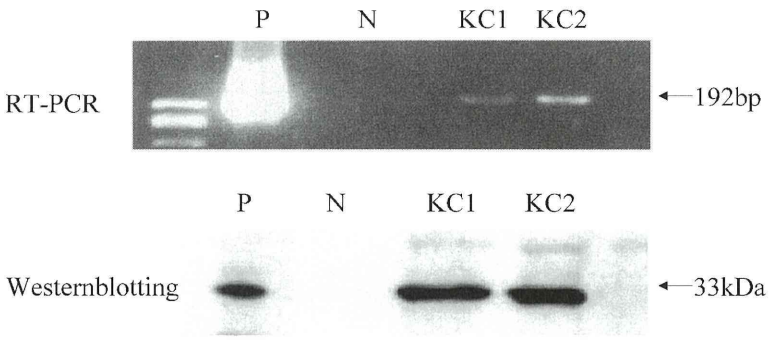

Fig. 1 RT-PCR and Western blot analysis of Fas ligand.
RT-PCR : Total RNA was isolated from fresh prepared rat Kupffer cells and subjected to RT-PCR. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The product of Fas ligand amplification migrated at the predicted size of 195 bp. Lane KC1 and KC2 were 2 rat Kupffer cells. Lane P and N were positive control and negative control, respectively.
A DNA marker is shown on the left.
Western blotting : Protein was isolated from 2 rat Kupffer cells. Anti rat FasL mAb was used to detect their protein levels on Western blot. Lane KC1 and KC2 were fresh prepared Kupffer cells from Wistar rats.
Fas ligand was expressed in rat Kupffer cells at mRNA and protein levels.

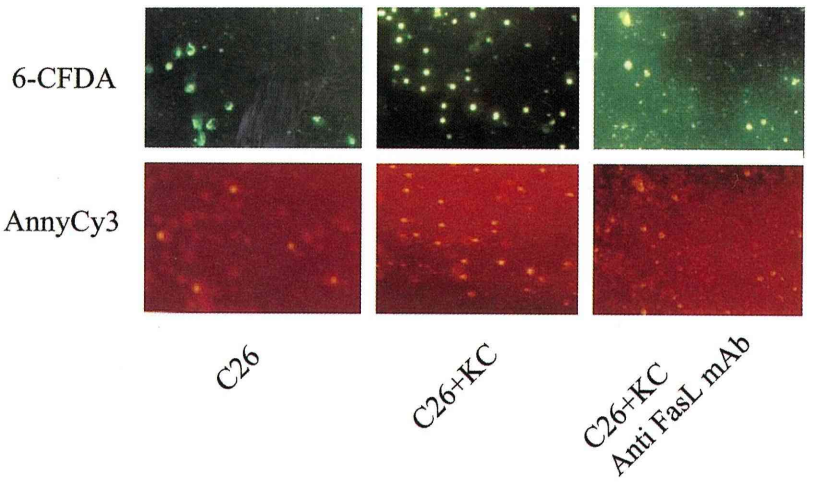

Fig. 2 Kupffer cells induce apoptosis in colon 26.
Colon 26 cells (2×10^5/ml) were incubated for 4 hrs with/without 100 ul supernatant of Kupffer cells (2×10^6/ml). 6-carboxyfluorescein diacetate (6-CFDA) was used to detect live cells. Annexin V-Cy3.18 (AnnyCy3) was used to detect necrotic cells and apoptic cells. Cells starting the apoptotic process stained both with AnnyCy3 and 6-CF. Colon26 cells staining both with AnnyCy3 and 6-CF were increased by Kupffer cell supernatant. Anti FasL mAb inhibited the increase by Kupffer cell supernatant in colon26 cells staining both with AnnyCy3 and 6-CF.

Cy3.18 and 6-carboxyfluorescein diacetate (CFDA), and then observed under fluorescence microscopy. Both annexinV-Cy3.18 and 6-CFDA appeared in color (Fig. 2). In other words, apoptosis was induced in colon 26 cells. Moreover, when anti-FasL mAb was added to this system, color expression of annexinV-Cy3.18 was suppressed.

3. Quantitative apoptosis assay

Kupffer cells were mixed with colon 26 cells labeled with BrdU and cultured at the E/T ratio of 1 : 10. When the BrdU concentration discharged in colon 26 cytoplasm was measured by ELISA, the colon 26 count was $1.4\times10^{-1}\pm0.19\times10^{-1}$ and the Jurkat count was $1.7\times10^{-1}\pm0.088\times10^{-1}$. In independent cultures of colon 26 and Jurkat cells, the colon 26 count was $1.1\times10^{-1}\pm0.04\times10^{-1}$ and the Jurkat count was $1.3\times10^{-1}\pm0.015\times10^{-1}$. Apoptotic index in colon 26 and Jurkat were both significantly high when Kupffer cells were added (Fig. 3). This means that apoptosis was induced by Kupffer cells, that DNA fragmentation was produced and that prior to cell destruction, the nucleus was destroyed and BrdU-labeled DNA drained off into the cytoplasm.

In inhibition experiments using anti - FasL mAb, goat IgG as the anti-FasL mAb isotype, was added to colon 26 and Jurkat cells. The colon 26 count was

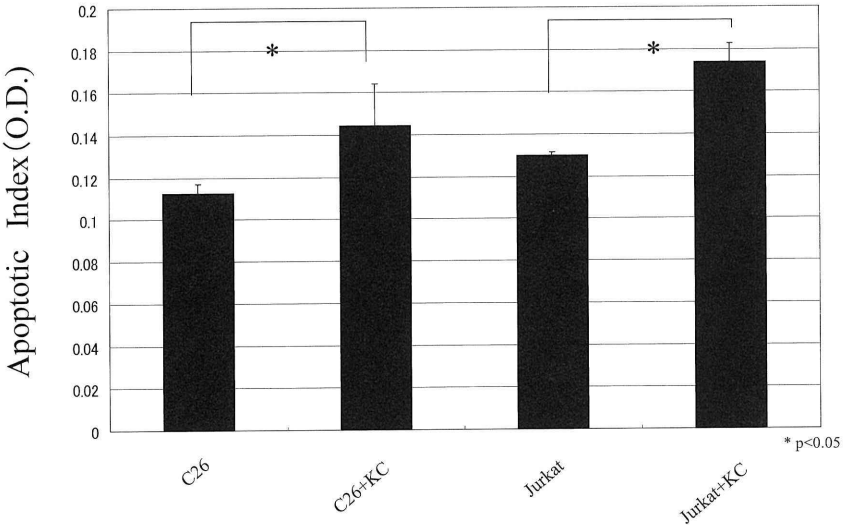

Fig. 3 Quantitation of apoptotic cancer cells induced by Kupffer cells. Cancer cells (colon26 and Jurkat) at the concentration of 2×10^5/ml were labeled with 1 mM BrdU overnight. The 2nd day, 1×10^4 of labeled cancer cells were placed into each well of a 96-well plate with/without 1×10^5 Kupffer cells. After 4 hrs, the plate were centrifuged and the supernatant discarded.
The cells were lysed and BrdU-labeled DNA was measured using an enzyme-linked immunosorbent assay (ELISA) kit from Roche (Mannheim, Germany)
N=4, paired Student's t-test, compared with cancer cells treated wuth Kupffer cells.

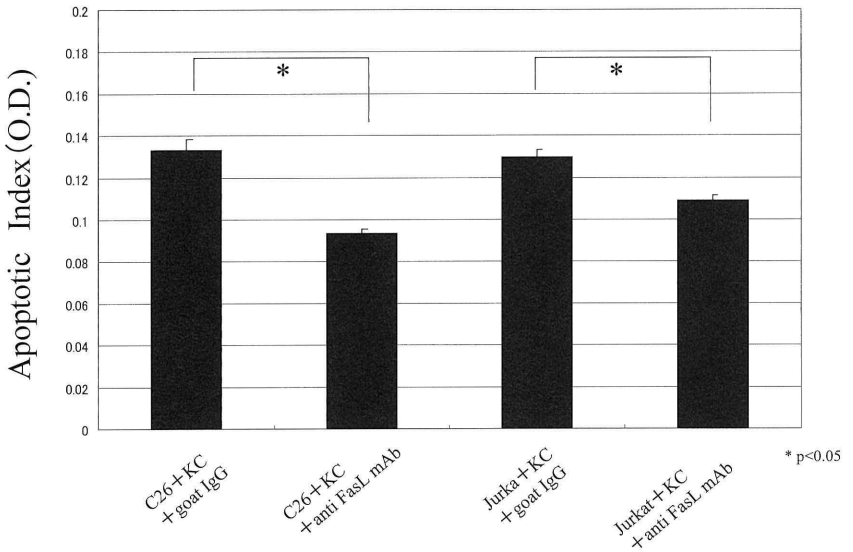

Fig. 4 Anti FasL mAb inhibited cancer cell apoptosis induced by Kupffer cells using an ELISA kit as in Fig. 3.

$1.3\times10^{-1}\pm0.043\times10^{-1}$ and the Jurkat count was $1.2\times10^{-1}\pm0.033\times10^{-1}$. On the other hand, when anti FasL mAb was added to colon 26 or Jurkat cells, the colon 26 count was $0.93\times10^{-1}\pm0.024\times10^{-1}$ and the Jurkat count was $1.09\times10^{-1}\pm0.002\times10^{-1}$. It was suspected that apoptosis was due to the Fas-Fas ligand system in which elution of BrdU-labeled DNA fragments into the cytoplasm was suppressed (Fig. 4).

Discussion

In colon cancer, the mechanism for metastasis to the liver is as follows. Cancer cells enter the bloodstream and adhere to the hepatic sinusoids via the portal vein system, initiating the formation of micro-metastasis[9,19]. When cancer cells reach the hepatic sinusoids, they first come in contact with vascular endothelial cells or Kupffer cells[5,20]. Hepatic Kupffer cells are macrophages that dwell in the sinusoids of the liver, and they contribute to immunity by such means as phagocytosis of wastes in the body, killing of pathogens, and secretion of various cytokines[21,22]. In 1998, Muschen et al. reported that Kupffer cells contribute to the immune mecha-

nism in that they express Fas ligand and induce apoptosis in Fas bearing cytotoxic T cells[15]. In our investigation, it was confirmed that rat Kupffer cells express Fas ligand at both the mRNA and protein levels.

We also looked to see if the Fas ligand of Kupffer cells actually functions and if apoptosis is induced in cancer cells. A Jurkat cell strain, which is a Fas-expression strain[23], was used as labeled cancer cell together with colon 26, which originates from colon cancer and can metastasize[24]. In preliminary experiments, it was confirmed by Western blotting that colon 26 expresses Fas protein. Kupffer cell culture supernatant was added to each cancer cell strain and an apoptosis detection kit was used to confirm apoptosis under fluorescence microscopy. In the apoptosis detection kit, annexinV labeled with Cy3.18 binds to phospholipid phosphatidylserin, which is expressed on the cell membrane early in apoptosis[25,26], and fluorescence is emitted. When Kupffer cell supernatant is added, there is a clear increase in colon 26 and Jurkat cells, which emit this fluorescence, but they were inhibited by anti-Fas ligand antibody. These findings show that the Fas ligand expressed in Kupffer cells functioned, and Kupffer cells induced apoptosis in both Jurkat and colon 26 cells by means of the Fas-Fas ligand system. In the present study, Kupffer cell supernatant was added for the following reasons. 1) In the detection of apoptosis, it is difficult to distinguish which cells have undergone apoptosis when two types of cell have been mixed together ; and 2) Fas ligand does not bind to Fas when cells contact each other[27]. Instead, it is thought that Fas ligands appearing on the cell membrane is shed and they bind to the Fas antigen of target cells,and then they are turning on the signal for apoptosis[27]. When a DNA fragmentation kit was used in the apoptosis assay, but only target cells were labeled with PO-labeled BrdU[28,29]. In order to assay BrdU eluted to cytoplasm by means of DNA plasmotomy, the apoptosis induction potential was measured in mixed culture. It was confirmed that Kupffer cells induce apoptosis in tumor cells. This suggests that in the case of solid cancer metastasis to the liver, after a small number of cancer cells have entered the liver, Kupffer cells might be able to prevent micro-metastasis via the Fas-Fas ligand system.

Nevertheless, in preliminary experiments, when the number of tumor cells was large in terms of E/T ratio, it was noted that apoptosis induction was suppressed. This implies that when metastasis has already progressed to a certain extent, or when a large number of tumor cells have already invaded the liver, Kupffer cells do not contribute to prevention via Fas-Fas ligand. Of course it is still unclear whether Kupffer cells prevent cancer cell invasion via active oxygen or NO.

Another possibility is that micro-metastasis can be suppressed by activating Kupffer cells.

Conclusion

It was confirmed that hepatic Kupffer cells express Fas ligand, and that *in vitro*, they induce apoptosis in tumor cells via Fas ligand, thus exhibiting an anti-tumor effect. It was also suggested that this could serve to suppress micro-metastasis of cancer cells in liver.

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大腸癌微小肝転移制御を目的とした肝 Kupffer 細胞の抗腫瘍効果の検討 (Fas/FasL システムを介して)

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【要旨】 肝 Kupffer 細胞は肝臓の類洞に存在するマクロファージであり、病原体の貪食機能、様々なサイトカイン等の分泌により免疫性に貢献している。近年、Kupffer 細胞が Fas ligand を発現し Fas bearing CTL(cytotoxic T lymphocytes) にアポトーシスを誘導することにより免疫メカニズムに貢献していると言う報告がなされた。今回我々は大腸癌の微小肝転移抑制因子としての Kupffer 細胞の働きに着目し、Kupffer 細胞が Fas/FasL システムを介して癌細胞にアポトーシスを誘導するか否かの研究を行った。1. RT-PCR 及び Western Blotting 法により、Kupffer 細胞での Fas ligand の発現を確認した。2. Kupffer 細胞との混合培養により、癌細胞株のアポトーシスが誘導された。3. 誘導された、癌細胞株のアポトーシス誘導能は抗 FasL 抗体により阻害された。この結果 Kupffer 細胞は Fas/FasL システムを介して癌細胞にアポトーシスを誘導し得ることが確認され、大腸癌微小肝転移抑制に影響しうることが示唆された。

〈**Key words**〉クッパー細胞、Fas/Fas リガンドシステム、大腸癌、微小肝転移