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Enhancement of Tumor Proliferation by Cyclosporine A in Early Phase of Experimental Hepatic Metastasis

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The effect of cyclosporine A (CsA) on *in vivo* growth of hepatic metastasis was studied. Murine colon 38 tumor cells (1×10^5) were inoculated via the superior mesenteric vein. Mice were grouped depending on CsA dosage and time schedules: Group A: CsA 30 mg/kg body weight on the 7, 8 and 9th post tumor inoculation days by gavage; Group B: CsA 15 mg/kg body weight 30 min before tumor inoculation and 2 times more at 24 h intervals; Group C: CsA 30 mg/kg body weight at the same dose timing as Group B. Measurement of the diameter of the largest tumor serially by weekly laparotomy up to 4 weeks revealed that the tumor growth rates were significantly greater in Groups B and C than those in Group A or the control (without CsA). The mean tumor doubling times in the control, and Groups A, B and C were 2.2±1.3, 2.0±0.5, 1.5±0.4 and 1.3±0.8 days, respectively. The mean tumor numbers of hepatic metastasis were 13.2±8.3, 11.3±7.3, 19.4±8.7 and 19.6±6.8, respectively. Values of tumor proliferation index obtained by bromodeoxyuridine immunohistochemistry were 10.0±6.1%, 14.9±8.0%, 28.6±8.2% and 30.1±12.4%, respectively, with significant differences (Groups B and C vs. A or control, $P<0.05$). *In vitro* MTT assay showed that cell viability rates were greater than 100% in the medium containing CsA concentrations of less than 10^{-7} mol/liter. However, a cytostatic effect of CsA was apparent at higher concentrations. In contrast to the previous *in vivo* finding of a cytostatic effect of CsA on tumor cells, we found a cytoproliferative action when CsA was administered early in the course of metastatic tumor implantation in the liver. The mechanism of cytoproliferative effect of CsA is unknown but may involve multiple factors.

Key words: Liver metastasis — Tumor growth — Colon 38 tumor cell — Immunosuppression — Cyclosporine A

Cyclosporine A (CsA) is a potent immunosuppressant, and its use in organ transplantation has considerably improved the survival of organ allografts.[1)] Its immunosuppressive action is attributed to the inhibition of $CD4^+$ T helper lymphocyte activation and interleukin 2 production.[2)] This has prompted investigation of its possible action on neoplastic cells. Indeed, an inhibitory effect of CsA on tumor cell progression has been documented not only in T-cell leukemia[3)] but also in some animal tumors.[4, 5)]

On the other hand, there is indirect evidence that is inconsistent with a cytostatic effect of CsA. In studies with partially hepatectomized animals, CsA enhanced hepatic regeneration.[6)] Similar hepatotrophic activity was also observed with a newer immunosuppressive drug, FK 506.[7)] In clinical transplantation, it is known that the recipients under immunosuppression with CsA are liable to a higher incidence of development of malignant diseases,[8)] and the growth of recurrent tumor was markedly accelerated after liver transplantation for hepatic malignancies in recipients treated with CsA immunosuppression.[9)]

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In view of these conflicting findings, we have investigated the effect of CsA on animal tumors, focusing particularly on metastatic capability. This experimental study has provided evidence of increased hepatic tumor metastasis and its proliferation following early administration of CsA.

MATERIALS AND METHODS

Mice and tumors Five- to six-week-old male C57B1/6J mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu. A mouse bearing colon 38 tumors (murine colon adenocarcinoma, MCA 38) was kindly supplied by Shionogi Pharmaceutical Co., Ltd., Osaka. The tumor had been maintained by subcutaneous implantation in the back of the animals every four weeks over 100 passages.

Tumor cell preparation and MTT assay Colon 38 tumor was minced and filtered through a #100 mesh filter. The tumor cells were treated with trypsin and suspended in RPMI-1640 at a density of 1×10^4 cells/ml. Viability of the cells was tested by the trypan blue dye exclusion method. Cells were maintained as a monolayer stock

culture at 37°C in a humidified atmosphere containing 5% CO_2 and were fed twice weekly with RPMI 1640 medium, supplemented with 10% fetal calf serum. The viability and survival of tumor cell *in vitro* were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay as described elsewhere, based on the original method by Mosmann.[10] Cells (1×10^4/100 μl/well) were plated into 96-well microtiter plates. They were cultured for 72 h, then 100 μl of medium containing the required concentration of cyclosporine or control vehicle was added and incubation was continued for another 24 h before assay. A mixture of MTT (4 mg/ml) and sodium succinate (0.1 mol/liter) in phosphate-buffered saline (PBS) was filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA) and 10 μl of solution/well was added, followed by incubation for a further 4 h at 37°C. Then 150 μl/well dimethyl sulfoxide was added to dissolve the formazan salt, and the plates were shaken for a few minutes. The absorbency of each resulting fluid was read on a model EAR easy reader (SLT-Labinstruments, Austria) at 540–630 nm. Survival rate was calculated as a percentage. The cells in control medium without MTT and sodium succinate were measured as blanks.

***In vivo* study of hepatic metastasis** Mice were anesthetized with ether inhalation. Through a small midline skin incision, one branch of the superior mesenteric vein was exposed. Then 0.1 ml of suspension containing 1×10^5 viable tumor cells was injected into the portal vein. Hemostasis was obtained by a gentle compression of the inoculation site with a cotton swab. At 2 weeks post tumor inoculation, the abdomen of each mouse was opened under the same inhalation anesthesia and explored for evidence of hepatic metastasis. If metastatic tumors were detectable, the number of metastases on the surface of the liver was counted and the size of the largest tumor was measured at its greateat diameter. Exploratory laparotomy was repeated at 3 weeks post tumor inoculation to count the number of detectable hepatic metastases and the size of the tumor which had been measured at the time of the first exploration. At 4 weeks post tumor inoculation, all of the animals were killed. The total number of metastases in the liver (which was cut and sliced), the size of the same tumor and the weight of the liver were measured. The specimens were subjected to microscopic examinations after routine hematoxylin and eosin staining.

Drug administration and grouping of the animals CsA (supplied by Sandoz Co., Osaka) solutions for oral administration were made by dissolving the drug in olive oil. Three groups of animals were used, as follows: Group A: CsA 30 mg/kg body weight on the 7, 8 and 9th post tumor inoculation days by gavage; Group B: CsA 15 mg/kg body weight 30 min before tumor inoculation and 2 times more at 24 h intervals; Group C: CsA 30 mg/kg body weight at the same dose timing at Group B. Control animals received an equivalent volume of olive oil (control group). CsA for *in vitro* assay was also kindly supplied by the same company that provided the form for intravenous use. CsA concentration in the whole blood from some representative animals was measured by high-pressure liquid chromatography. The 12 h trough level of CsA after the last dose was in the range of 60.5–165.0 ng/ml for Group B and 95.3–284.5 ng/ml for Groups A and C.

BrdUrd immunohistochemistry In some of the animals in each group, bromodeoxyuridine (BrdUrd) 100 mg/kg in normal saline was inoculated intraperitoneally on the 7th post tumor inoculation day. These animals were killed and the livers were excised, immediately followed by fixation in 70% ethanol. Sections were made and stained by the avidin-biotin-peroxidase complex method using a Histofine SAB-PO (M) kit (Nichirei Corp., Tokyo). The labeling index of the tumor was expressed as the ratio of the number of the cells positively stained to that of the entire cells, determined by light microscopic examination of over 2,000 cells.

Statistics Data were expressed as the mean ± standard deviation of the mean. ANOVA was used for comparison of the mean values between the groups. A P value of less than 0.05 was considered significant.

RESULTS

MTT assay The results of MTT assay to determine the *in vitro* effect of CsA on colon 38 tumors at various

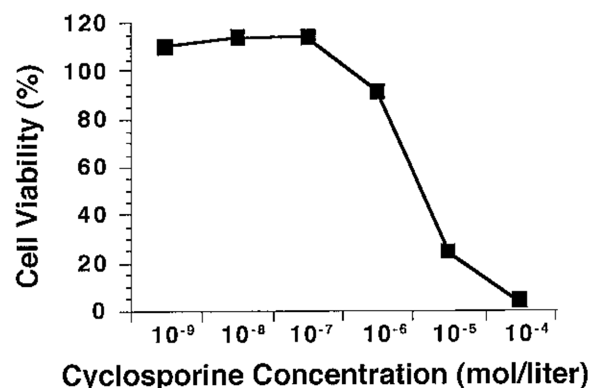

Fig. 1. MTT assay for colon 38 tumor cells exposed to different concentrations of CsA *in vitro*. Cell viability rate expressed as percent of that in the control medium without CsA is plotted against CsA concentration. Cell viability rates tend to be greater than 100% in the medium with CsA concentrations of 10^{-9}–10^{-7} mol/liter (P=0.02). Rapid loss of viability occurred at CsA concentrations greater than 10^{-6} mol/liter.

concentrations are shown in Fig. 1. Cell viability was 111.3%, 113.0% and 113.6% in the medium containing 10^{-9}, 10^{-8} and 10^{-7} mol/liter, respectively as compared to the control. The differences were, however, not statistically significant (P=0.2). However, the viability fell sharply at higher CsA concentrations (91.8%, 23.1% and 2.7% for CsA concentrations of 10^{-6}, 10^{-5} and 10^{-4} mol/liter, respectively).

***In vivo* tumor growth of hepatic metastasis** Gross inspection of hepatic metastases showed various sizes of tumors maintaining nearly spherical shape throughout the observation period. Metastatic tumors in cut specimens of the liver were preferentially distributed in the peripheral surface area of the liver. The distribution pattern of the metastatic foci in the liver did not differ significantly among the groups. Under microscopic observation, the tumors invariably showed central necrosis when they were greater than 2 mm in diameter. Changes of the mean of the greatest diameter measured at 2, 3 and 4 weeks post tumor inoculation are shown in Fig. 2. At 2 weeks, the mean tumor diameter in Group B (1.5±0.5 mm) was significantly greater than that of the control group (0.7±0.5 mm, $P<0.01$). At 3 weeks, tumor diameter in Group B (4.8±2.0 mm) was greater than that of the control (2.0±1.3 mm, $P<0.05$) or that of Group A (2.8±1.1 mm, $P<0.001$). The tumor diameter in Group C (4.4±1.3 mm) was also greater than that of the control group ($P<0.001$) or that of Group A ($P<0.001$). At 4 weeks, the tumor diameter of Group B (11.2±4.9 mm) was greater than that of Group A (6.6 ± 3.5 mm, $P<0.05$). The tumor diameter of Group C (13.4±4.8 mm) was greater than that of the control group (8.9± 4.7 mm, $P<0.05$) or that of Group A ($P<0.01$). Values of tumor doubling time in days calculated by using the formula reported by Schwartz[11] during the observation period were 2.2±1.3, 2.0±0.5, 1.5±0.4 and 1.3±0.8, but the differences were not significant. The numbers of hepatic metastases detectable by inspection at each time point are shown in Table I. A steady increase in tumor number was noted. Although tumor number significantly increased at the termination, possibly due to counting of all the tumors after cutting the liver, most of the tumors were located at the surface of the liver and were countable by surface inspection. Generally, more tumors were

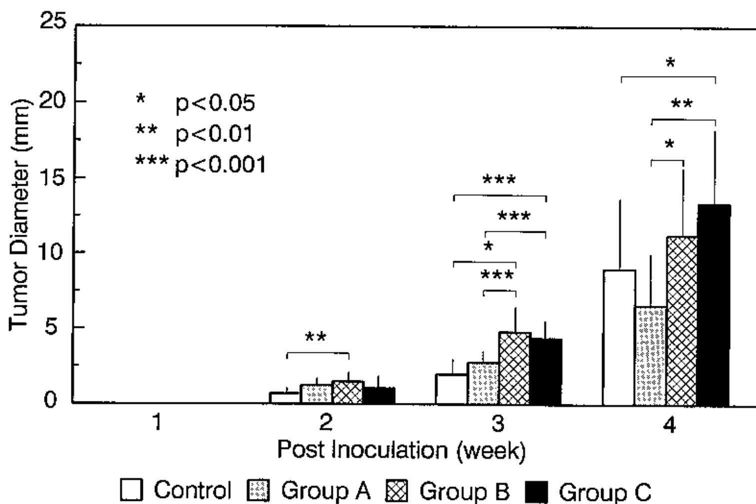

Fig. 2. Growth of colon 38 tumors metastasizing to the liver in relation to the time post tumor inoculation. The mean of the greatest tumor diameter of the largest metastatic nodule is shown by the height of the columns for the control, and Groups A (CsA 30 mg/kg body weight on the 7, 8 and 9th post tumor inoculation days), B (15 mg/kg of CsA given 30 min before inoculation and on post inoculation days 1 and 2) and C (same dosage of CsA as in Group A at the same time points as in Group B). Asterisks indicate statistically significant differences between the groups.

Table I. Number of Colon 38 Hepatic Metastatic Tumors

Group	Tumor number/liver		
	2 week	3 week	4 week
Control (n=11)	1.5±0.9	2.8±2.1	13.2±8.3
Group A (n=9)	2.3±1.7	3.6±2.2	11.3±7.3
Group B (n=10)	3.3±1.7	4.3±1.7	19.4±8.7
Group C (n=11)	2.7±1.2	5.5±2.0	19.6±6.8

* $P<0.01$, ** $P<0.05$

formed in Groups B and C than in Group A or in the control group (significant differences).

Tumor proliferation labeling index The values of labeling index of the tumor cells, represented as the ratio of BrdUrd-positive cells at 2 weeks post tumor inoculation, were 10.0±6.1%, 14.9±8.0%, 28.6±8.2% and 30.1±12.3% for the control, and Groups A, B and C, respectively. Approximately 30% of the tumor cells in Groups B and C were BrdUrd-positive cells, indicating DNA-synthesizing cells, whereas less than 15% of the tumor cells in Group A and the control group were positive. These differences were significant ($P<0.01$ for the control vs. Group B and Group A vs. Group B, C; $P<0.05$ for the control vs. Group C). The labeling index of normal hepatocytes was less than 1% in all of the specimens examined. There was no significant difference in the labeling index of the normal hepatocytes among the groups.

DISCUSSION

CsA is a fungal metabolite with potent immunosuppressive activity, and has been widely used in the field of clinical transplantation.[1] However, it has a wide spectrum of side effects, including not only those secondary to its cytotoxic effect on T helper lymphocyte, but also those seemingly arising from non immunological mechanisms. One of the latter is hypertrichosis, which prompted an *in vivo* study on the cytoproliferative effect of CsA on epidermal keratinocytes.[12] Contrary to the initial impression of proliferative action, accumulating evidence suggests that CsA exerts cytostatic activity on various normal and tumor cells.[3, 4, 13, 14] Saydjari *et al.* reported an antitumor effect of CsA using murine colon tumor 26 implanted subcutaneously.[5] They proposed that CsA inhibits ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis. However, the result was not reproducible in subsequent *in vivo* T-cell tumor model studies by other investigators.[15]

The results of the present study showed that CsA enhanced proliferation of hepatic metastasis *in vivo*. In the *in vitro* study, CsA expressed a biphasic pattern of effects, being cytostatic at concentrations greater than 10^{-6} mol/liter, but cytoproliferative at lower concentrations in MTT assay, although the differences were not significant ($P=0.02$). CsA concentrations in the blood of the animals were maintained in the range employed clinically after organ transplantation (the 12-h trough level of cyclosporine in the whole blood of transplant recipients is usually 1×10^{-6} to 2×10^{-6} mol/liter). These levels are compatible with the cytostatic range in the *in vitro* study. This may imply that CsA concentration in the tissue is actually lower than that in the blood. Another possibility is that diverse *in vivo* cellular interactions of the tumor cells are not reproduced in the *in vitro* study. Also, liver-generated metabolites can exhibit subtly different properties from the native molecule.[16]

Experience in liver transplantation for hepatocellular carcinoma has clearly demonstrated that the recurrent tumors grow much faster in immunosuppressed patients than in non-immunosuppressed patients.[9] The immunological basis for this interesting observation needs to be elucidated. It has long been suspected that suppression of immunosurveillance of tumor cells is depressed in immunocompromised hosts.[17] However, no experimental work has been done to prove this. Perhaps more important is the clinical observation in liver transplantation that the recurrent tumor appears to originate from tumor cells implanted in the new liver at the time of transplantation. It has been postulated that manipulation of the liver at the time of hepatectomy can disperse tumor cells in the blood circulation, and they subsequently return to the transplanted liver, forming metastases.[18] This indicates that the time of transplantation is a critical moment for establishing metastasis, since that is the very time when immunosuppression treatment is begun. However, no good model is available to investigate this interesting subject. One problem in establishing such a model is that biological characteristics can differ from tumor to tumor. With colon 26 tumor, which is a relatively fast-growing murine colon carcinoma, our preliminary investigation failed to find a proliferative effect of CsA in *in vitro* MTT assay. Another point is the site specificity of the growth pattern of tumor cells. It is reasonable to assume that the biological behavior of the tumor is different when it is implanted in a non-vascularized organ such as subcutaneous tissue than in a vascularized organ as the liver. Indeed, colon 38 tumor itself progresses differently depending on the site of tumor implantation.[19]

The process of cancer metastasis to the liver is considered to consist of a long series of sequential interrelated steps which include a) growth and invasion of the primary tumor into the venules and/or capillaries, b) transport of the malignant cells in the circulation, c) arrest in the liver and finally d) growth and invasion as a metastatic tumor.[20] The present experiment was designed exclusively for investigation of processes c) and d). Therefore, the cytoproliferative action of CsA is a result of either its enhancing potential on tumor cell arrest and/or its direct effect on DNA synthesis and cell replication. Moreover, the fact that CsA administration 7 days after tumor inoculation failed to show any obvious proliferative effect signifies the importance of events which occur early in the course of tumor implantation.

The hypothesis that CsA potentiates tumor cell arrestability in the liver is supported by the observation that more metastatic hepatic tumors are found in the animals treated with CsA than in those without CsA. This is

consistent with the classical view that the number of metastases is proportional to the number of tumor emboli arrested within the capillary bed of an organ.[21] It has long been recognized that the majority of intravenously injected tumor cells are mortally damaged and fail to form metastasis.[22] It is conceivable that CsA inhibits this killing process of the tumor cells in the circulation.

There is considerable evidence that CsA increases the number of tumor emboli. CsA is known to be vasoconstrictive, affecting the smooth muscle of vessels[23] so that clumps of tumor cells can be arrested as they traverse the narrowed lumen of the vessels. Anatomically, metastatic tumors have a predilection for the acinar zone one area in the liver.[24] This seems inconsistent with increased tumor emboli due to vasoconstriction, since the zone one is lacking in small arteries or veins. Nevertheless, the chance of tumor cell arrest in the sinusoids is greatly enhanced when the proximal venules are narrowed by vasospasm or microembolism. Another possible mechanism by which CsA enhances tumor cell arrestability is interaction with prostanoids, particularly thromboxane. Thromboxane A_2 is a vasoconstrictive agent, but more importantly, it increases leukocyte adhesion to endothelial cells.[25] Clinical experience with kidney transplantation has shown that CsA administration causes a significant elevation of thromboxane B_2, a stable metabolite of thromboxane A_2, in the plasma.[26] Increased thromboxane in the blood leads to not only alteration of the microvascular circulation, but also damage to the endothelial cells and further recruitment of platelets to aggregate.[27] Moreover, interaction between platelets, leukocytes and tumor cells can induce production of various cytokines and other chemoactive substances.[28] This results in increased adhesive interaction of the tumor cells with endothelial cells, possibly by up-regulation of surface adhesion molecules.[29] We have preliminary data indicating that animals given CsA have increased thromboxane B_2 in the blood (data not shown).

It is conceivable that all of these changes contribute to a situation which favors tumor cell arrest in the sinusoids of the liver. Further, aggregation of the platelets protects tumor cells not only from mechanical damage but also from immunological attack of the host. It has been shown that tumor cell adhesion to endothelial cells can protect the tumor cells from natural killer cell-mediated lysis.[30]

A possible cytoproliferative role of CsA as evidenced by both *in vitro* and *in vivo* studies is consistent with well known observations that CsA administration in partially hepatectomized animals induces enhanced liver regeneration.[6] This effect as a growth factor has also been demonstrated with FK506, a newer immunosuppressive agent which has a similar chemical structure and pharmacological characteristics to CsA.[7] Various other immunosuppressive drugs have similar hepatic growth effects.[31] However, the mechanism of the hepatocyte-stimulatory effect of these drugs is not fully understood.

In conclusion, the characteristic feature of the cytoproliferative action of CsA as shown in the present study can be explained by its site specificity for liver. The classical theory of seed and soil for tumor metastasis seems applicable.[32] It is speculated that CsA can alter the nature of the seed (cancer cell) itself by its action of increasing cell mitotic activity. Further investigation with a genetic approach may prove informative. In the liver, as the soil, CsA may affect the environment for circulating tumor cells in such a way as to facilitate metastasis. Thus, it is considered that tumor growth involves specific interactions between the metastatic cells and the organ environment, possibly through specific binding to endothelial cells and responses to local growth factors. Whatever the mechanism of CsA involved in hepatic tumor metastasis is, it must be multifactorial. CsA is a potent immunosuppressant which contributes to the present success of organ transplantation. However, the use of this drug must be judicious when it is used as an adjunct to liver transplantation in patients with malignant tumors.

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