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Immunosuppressive drugs and their effect on experimental tumor growth

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Abstract The effect of cyclosporin (CyA), FK 506, and mycophenolate mofetil (MPM) on tumor growth was investigated using syngeneic mouse colon carcinoma 38. Mice were laparotomized and the tumor cells were injected into the portal vein to establish liver metastasis. The animals were grouped as follows: groups A-1, B-1, and C-1 were given CyA [15 mg/kg body weight (BW)], FK 506 (0.15 mg/kg BW), and MPM (100 mg/kg BW), respectively, 30 min before tumor inoculation and daily for 5 days by gavage; groups A-2, B-2, and C-2 were given CyA (30 mg/kg BW), FK 506 (0.3 mg/kg BW), and MPM (200 mg/kg BW), respectively, with the same dose timing; and groups A-3, B-3, and C-3 received CyA (30 mg/kg BW), FK 506 (0.3 mg/kg BW), and MPM (200 mg/kg BW), respectively, on the 7th post-tumor inoculation day and on the following 5 days. The mean tumor diameter in groups A-1 and A-2 was greater than that in the control group and in groups C-1 and C-2 at 3 weeks ($P < 0.05$). The mean tumor numbers in groups A-1 and A-2 were greater than those in the control group and in groups C-1 and C-2 at 4 weeks ($P < 0.05$). With in vitro MTT assay, all three drugs acted cytostatically on tumor cells with a higher concentration (10^{-6}–10^{-4} mol/l), while no cytostatic effect was noted with CyA at a lower concentration (10^{-9}–10^{-7} mol/l). Labeling indexes (%) by bromodeoxyuridine (BrdUrd) immunohistochemistry in groups A-1, A-2, and B-1 were significantly greater than those in the control group and in groups C-1 and C-2 ($P < 0.05$). Although the mechanism of cytoproliferative action of CyA and FK 506 is not well understood, a decrease in immunosurveillance capability by natural kill cells due to suppression of interleukin-2, their direct action as growth factors, and/or enhanced tumor cell adhesion can be considered.

Key words Immunosuppression, tumor growth · Tumor growth, immunosuppression · Cyclosporin, tumor growth · FK 506, tumor growth · Mycophenolate, tumor growth

Introduction

One of the most interesting subjects in tumor biology is that of host-tumor interaction. Of particular interest is the behavior of the tumor in an immunosuppressed host. Undoubtedly, depression of the host's cellular immunity creates a situation in which the host can easily accept heterogeneous tumor implantation, a common phenomenon in athymic nude mice with xenotransplantation. Moreover, depressed cellular immunity tends to result in the development of de novo malignancy in the host, as seen in AIDS patients with Kaposi's sarcoma or lymphoma.

In clinical transplantation, the state of immune depression is artificially created with the use of various immunosuppressants. It is well known that these patients develop de novo malignancies more often than those without immunosuppression [16]. Of interest is the finding that in patients with hepatic malignancy who undergo liver transplantation, recurrent tumors grow at a much faster rate than those in patients without immunosuppression [28]. Although clear explanations for this phenomenon have not yet been provided, there are two important factors that may be involved. One is an immune factor that affects tumor growth due to suppressed immunity in the host. The other factor is a direct pharmacologic effect of the drugs on tumor cells in cytoproliferative fashion.

This study was designed to investigate the effect of various kinds of immunosuppressive drugs on tumor proliferation using in vitro and in vivo models of experimental hepatic metastasis.

Materials and methods

Mice and tumors

For the animal experiments, the "Principles of laboratory animal care" (NIH Publication No. 85-23, revised 1985) were followed, as well as the regulations of the Animal Research Laboratory of Nagoya University, School of Medicine. Five-to six-week-old male C57B1/6J mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan). A mouse bearing colon 38 tumors, syngeneic murine colon adenocarcinoma, of which the original tumor line was obtained by chemical induction with dimethylhydralazine in C57B1/6J mice [4], was kindly supplied by Shionogi Pharmaceutical (Osaka, Japan). It had been maintained by subcutaneous implantation in the back of the animals every 4 weeks in more than 100 times of passage.

Immunosuppressive drug

Cyclosporin A (CyA) was obtained from Sandoz (Osaka, Japan), FK 506 from Fujisawa Pharmaceutical (Osaka, Japan), and mycophenolate mofetil (MPM) from Sytex (Tokyo, Japan).

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 with the trypan blue dye exclusion method. Cells were maintained as 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 cells in vitro were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide assay (MTT; Sigma), as previously described [22]. 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 concentrations of various immunosuppressive drugs or control vehicles was added to incubate for another 24 h before the assay. A mixture of MTT (4 mg/ml) and sodium succinate (0.1 mol/l) in phosphate-buffered saline (PBS) was filtered through a 0.45-µm membrane filter (Millipore, Bedford, Mass., USA) and 10 µl solution/well was added, followed by incubation for an additional 4 h at 37 °C. Then, 150 µl/well dimethyl sulfoxide was added to dissolve the formazan salt, and plates were shaken for a few minutes. The absorbency of each resulting fluid was read on a model EAR easy reader (SLT-Lab instruments, Austria) at 540–630 nm. Survival rate was calculated as a percentage. The cells in the control medium without the mixture of 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 of the branches 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 gently compressing 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. When metastatic tumors were detectable, the size of the largest tumor was measured for its greatest diameter. Exploratory laparotomy was repeated at 3 weeks post-tumor inoculation to inspect and measure the same tumor that had been measured at the time of the first exploration. Four weeks post-tumor inoculation, all of the animals were sacrificed. The total number of metastases of the liver was counted by slicing the specimen for detailed examination. The specimens were examined microscopically with routine hematoxylin and eosin staining.

Drug administration and grouping of the animals

The following ten groups of animals ($n = 12$ in each group) were identified, depending on the dose of the drug and timing of its administration: groups A-1, B-1, and C-1 were given CyA [15 mg/kg body weight (BW)], FK 506 (0.15 mg/kg BW), and MPM (100 mg/kg BW), respectively, 30 min before tumor inoculation and daily for 5 days by gavage; groups A-2, B-2, and C-2 were given CyA (30 mg/kg BW), FK 506 (0.3 mg/kg BW), and MPM (200 mg/kg BW), respectively, with the same dose timing; and groups A-3, B-3, and C-3 received CyA (30 mg/kg BW), FK 506 (0.3 mg/kg BW), and MPM (200 mg/kg BW), respectively, on the 7th post-tumor inoculation day and on the following 5 days. The dosage of each drug was determined on the basis of previous experience in experimental and clinical allograft transplantation and was within the range providing effective immunosuppression [1, 8, 14]. Control animals received an equivalent volume of vehicle (control group). In some of the animals in each group, the drug levels in whole blood were measured.

BrdUrd immunohistochemistry

In additional animals ($n = 5$ in each group), bromodeoxyuridine (BrdUrd), 100 mg/kg in normal saline, was injected intraperitoneally on the 12th post-tumor inoculation day. These animals were sacrificed 60 min after injection of BrdUrd and the livers were excised immediately, followed by fixation in 70 % ethanol. The sections were made and they were stained following the avidin biotin peroxidase complex method, using a Histofine SAB-PO (M) kit

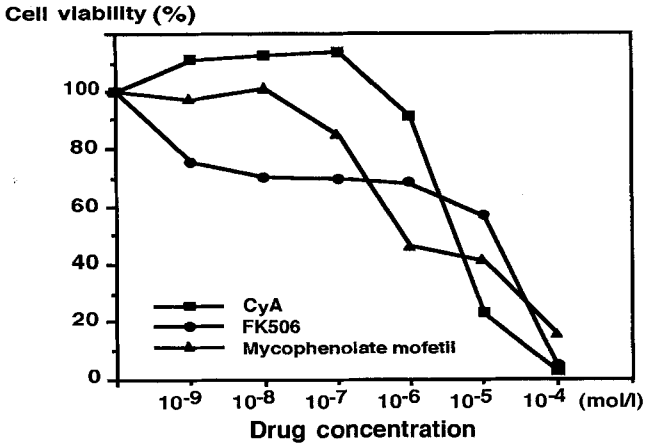

Fig. 1 MTT assay of colon 38 with CyA, FK 506, and MPM. Cell viability decreased sharply with high drug concentrations (10^{-6}–10^{-4} mol/l) in all three drugs. However, with CyA, cell viability was well maintained in its low concentration (10^{-9}–10^{-7} mol/l)

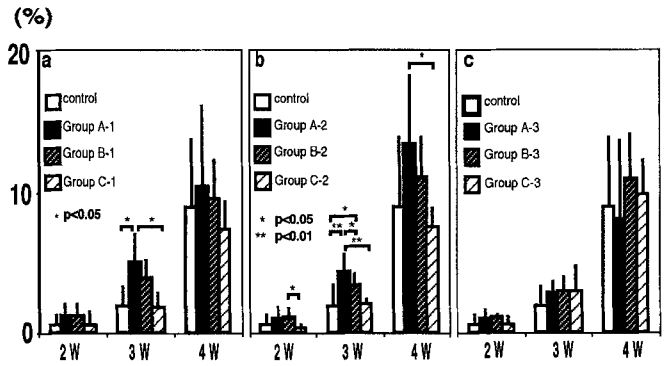

Fig. 2 a–c Change in metastatic tumor size after tumor inoculation in the liver via the portal vein with: **a** CyA, 15 mg/kg body weight (group A-1) and FK 506, 0.15 mg/kg body weight (group B-1), administered 30 min before tumor inoculation and daily for the following 5 days by gavage. Tumor size increased significantly more than in control animals or in those with MPM, 100 mg/kg body weight (group C-1) at the same dosing schedule as the other two drugs; **b** when the dosages of each drug were doubled (groups A-2, B-2, and C-2), the differences in tumor size were more prominent between the groups with CyA and FK 506 vs control and MPM; **c** however, no significant difference in tumor size was noted between the groups that received immunosuppressive drugs after 1 week of post-tumor inoculation (groups A-3, B-3, and C-3)

(Nichirei, Tokyo). Labeling index of the tumor was expressed as the ratio of the number of cells positively stained to the total number of cells examined light microscopy.

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 the MTT assay on colon 38 tumors for the in vitro effect of CyA, FK 506, and MPM are shown in Fig. 1. The rates of cell viability in different concentrations of these drugs were 111.3 %, 113.0 % and 113.6 % in the medium with CyA, 75.6 %, 70.2 %, and 70.0 % with FK 506, and 97.4 %, 101.2 %, and 85.0 % with MPM in drug concentrations of 10^{-9}, 10^{-8}, and 10^{-7} mol/l, respectively. The viability of the cell fell sharply with greater concentrations: 91.8 %, 23.1 %, and 2.7 % for CyA; 68.2 %, 57.0 %, and 4.7 % for FK 506, and 46.2 %, 41.3 % and 16.0 % for MPM in concentrations of 10^{-6}, 10^{-5}, and 10^{-4} moll, respectively.

In vivo tumor growth of hepatic metastasis

The mean concentration 30 min after oral administration of CyA (group A-1) and of FK 506 (group B-1) in whole blood were 560.5 ± 75.5 ng/ml and 13.4 ± 7.4 ng/ml, respectively. The mean concentration of MPM (group C-1) in the serum was 141.3 ± 10.4 μg/ml. Typical findings of hepatic metastases, by gross inspection, showed that varying sizes of tumors grew, maintaining a nearly spherical shape throughout the observation period. Metastatic tumors in the cut specimens of the liver at the time of sacrifice 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 tumor invariably formed a central necrosis when the average size of the tumor exceeded 2 mm in diameter. There was minimal reaction of inflammatory cells in or around the tumor. Except for the animals treated with MPM, all animals developed metastatic tumors. The incidence of hepatic metastasis in groups C-1, C-2, and C-3 was 81.8 %, 72.2 % and 100 %, respectively.

Changes in the size of the tumors in each group of animals were serially measured at 2, 3, and 4 weeks post-tumor inoculation and are shown in Fig. 2. At 2 weeks post-tumor inoculation, the mean tumor diameter in group B-2 (1.2 ± 0.5 mm) was significantly greater than that in group C-2 (0.4 ± 0.2 mm; $P < 0.05$). At 3 weeks, the mean tumor diameter in group A-1 (4.8 ± 1.9 mm) was greater than that in the control group (2.0 ± 1.3 mm) and in group C-1 (1.8 ± 0.6 mm; $P < 0.05$). The mean tumor diameter in group A-2 (4.4 ± 1.3 mm) was greater than that in the control group ($P < 0.01$) and in groups B-2 (3.4 ± 0.7 mm; $P < 0.01$) and C-2 (2.0 ± 0.4 mm; $P < 0.01$). The mean tumor diameter in group B-2 was also greater than that in the control group ($P < 0.05$). A steady increase in the mean diameter of the tumor was also noted in groups A-3, B-3, and

C-3, although the differences among the groups were not significant at each time point.

The mean numbers of detectable hepatic metastases 4 weeks post-tumor inoculation in group A-1 (19.3 ± 8.7) and A-2 (19.6 ± 6.8) were significantly greater than those in the control group (13.2 ± 8.3) and groups C-1 (12.6 ± 6.6) and C-2 (8.6 ± 5.6; $P < 0.05$). The mean number of tumors in group B-2 (18.5 ± 3.8) was also significantly greater than that in C-2 ($P < 0.05$).

Tumor proliferation labeling index

Labeling indexes of the tumor cells, represented as the ratio of BrdUrd-positive cells on the 12th day of post-tumor inoculation to the total cells examined, were 10 % ± 6.1 %, 30.0 % ± 8.9 %, 15.2 % ± 5.1 %, and 8.0 % ± 4.1 % for control, A-1, B-1, and C-1, respectively. In groups A-2, B-2, and C-2, they were 28.6 % ± 8.2 %, 14.9 % ± 5.1 %, and 8.5 % ± 4.3 %, respectively. In groups A-3, B-3, and C-3, they were 14.9 % ± 8.0 %, 12.1 % ± 7.1 %, and 10.5 % ± 6.5 %, respectively. Significant differences were noted between the control croup, C-1, or C-2 and A-1, A-2, or B-1 ($P < 0.05$). 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

Three distinctly different and characteristic immunosuppressants – cyclosporin (CyA), FK 506, and mycophenolate mofetil (MPM) – were used to investigate their effects on tumor cell growth. Both CyA [3] and FK 506 [8] are potent immunosuppressants that have similar chemical structures and biological properties. The main pharmacological action of these two drugs has been attributed to the inhibition of $CD4^+$ T helper lymphocyte activation and interleukin-2 production. This obvious cytotoxic effect on lymphoid cells has prompted investigation of their possible effects on neoplastic cells. Indeed, the inhibitory effect of CyA on tumor cell proliferation has been documented, not only in T-cell leukemia [26] but also in some animal tumors [6, 13, 23]. Experimental studies on animal T-cell leukemia have shown that CyA may inhibit deoxycarboxylate enzyme on purine metabolism [21], although investigations by others have failed to confirm this [23].

In contrast to the cytostatic capability of CyA, there has been some indirect but sufficient evidence that CyA has a cytoproliferative effect on certain non-neoplastic cells. In studies with partially hepatectomized animals, both CyA and FK 506 showed the distinctive property of promoting hepatic regeneration, as if they were hepatic growth factors [5, 11]. Based on the clinical observation that patients with CyA for immunosuppression often develop hypertrichiosis, it has been shown experimentally that CyA acts cytoproliferatively on keratinocytes [20]. Interestingly, however, despite the structural and chemical similarities between CyA and FK 506, hypertrichiosis has not been observed in patients receiving FK 506. As mentioned in the introductory part of this article, a perhaps more important clinical observation is that after liver transplantation for patients with hepatocellular carcinoma, recurrent tumor grow at a much faster rate than those in patients without immunosuppression [28]. However, the underlying mechanism of this interesting phenomenon is not clearly understood.

Although our in vitro study showed that all three of the drugs demonstrated a cytotoxic effect on the tumor cells at a high concentration, cell viability was relatively well maintained in the range of 10^{-7}–10^{-8} mol/l. Importantly, these lower ranges of drug concentrations correspond to the blood concentrations of the animals. In contrast to the in vitro study, CyA and FK 506 acted cytoproliferatively on tumor cells when they were administered at the time of tumor cell inoculation in in vivo settings.

The cytoproliferative action of CyA and FK 506 on neoplastic cells in vivo may be due to their depressive effect on cell-mediated immunity, as they induce inactivation of interleukin-2 [19], which subsequently induces suppression of immune surveillance of tumor cells [25]. Natural killer cells seem to play a major role in this mechanism as they can attack the circulating tumor cells when they are injected into the portal vein, thus preventing implantation of the cells in the liver [12]. An important observation in clinical liver transplantation is that a recurrent tumor likely originates from the tumor cells implanted in the new liver at the time of transplantation [15]. It has been postulated that manipulation of the native liver at the time of hepatectomy can disperse tumor cells in the blood circulation that return to the transplanted liver, forming metastatic foci [28]. This indicates that the time of transplantation is a critical moment in establishing metastasis, since that is the very time when immunosuppressive treatment is begun. Other such potential cells may include leukocyte-activating killer cells and pit cells in the liver, the latter of which is considered to be in the killer cell category [2].

In contrast to CyA or FK 506, MPM did not increase tumor growth. Moreover, MPM significantly decreased the chance of tumor metastasis in the liver. It is known that MPM selectively inhibits T and B cells and that its immunosuppressive property is different than that of CyA or FK 506. This is ascribed to the fact that MPM does not inhibit the production of IL-1, the production of IL-2, or the expression of the IL-2 receptor [17].

Nonimmunological mechanisms may also be involved in the enhancement of tumor growth with CyA

or FK 506. Both of these drugs, particularly CyA, are known to be vasoconstrictive substances affecting the smooth muscle of the vessels [7], which can increase the chance of trapping the clumps of tumor cells when they traverse the narrowed lumen of the vessels [27]. Tumor adhesion can be enhanced by thromboxane A_2, a vasoconstrictive agent and chemoattractant that increases leukocyte adhesion to endothelial cells [9]. Moreover, interaction between platelets, leukocytes, and tumor cells can induce the production of various cytokines and other chemoactive substances [18]. This results in increased adhesive interaction of the tumor cells with endothelial cells, possibly by upregulation of surface adhesion molecules [10]. Thus, the probability of a direct effect of CyA and FK 506 on neoplastic cells as growth factors is equivocal.

The mechanism of hepatic tumor metastasis associated with immunosuppressive drugs must be multifactorial. CyA and FK 506 are potent immunosuppressants that have contributed to the present success of organ transplantation. However, the use of these drugs must be judicious when they are used for liver transplantation in patients with malignant tumors. In such cases, combined administration with other types of immunosuppressive drugs, such as MPM, may lessen the possible cytoproliferative action of the conventional drugs on recurrent tumors.

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