

Inhibitory effects of adrenomedullary hormone on the induction and growth of fibrosarcoma by methylcholanthrene

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Abstract

OBJECTIVE: Effects of adrenomedullary hormone(s) on the induction and growth of fibrosarcoma by methylcholanthrene (MC) were examined.

METHODS: At 28 days of age, male Wistar rats were divided into four groups: control, 2) bilateral adrenomedullectomy (Bil. AMX), 3) right AMX + left adrenomedullary autotransplantation (AMX + AMT), 4) Bil. AMX + epinephrine injection (Bil. AMX + E) groups. 14 days after surgery, MC crystals were inserted underneath the dorsal skin, and in the Bil. AMX + E group, epinephrine was injected subcutaneously, twice every week.

RESULTS: The incidence of tumor at 90 days after the MC injection was 8 per 35 cases (22.9%) in the control group, 12 per 36 cases (33.3%) in the AMX + AMT group, 8 per 28 cases (28.6%) in the Bil. AMX + E group, and each value was lower compared with that of the Bil. AMX group, 24 per 34 cases (70.6%), ($P < 0.001$, $P < 0.002$, $P < 0.005$). Such differences among groups were not seen at 165 days after the injection of MC.

DISCUSSION: The mechanisms of effects of AMX, AMT and/or epinephrine on the tumor incidence have been discussed with reference to tumor promotion, vascular neoplasia, etc. Since norepinephrine remaining in the blood of AMX rats was ineffective, at least it is likely that this inhibitory effect of epinephrine is mediated via the β_2 -receptor.

CONCLUSION: The results suggest that adrenomedullary hormone, probably epinephrine, has inhibitory effects on the induction and growth of fibrosarcoma by MC, particularly in the early stage.

Introduction

It has been well known that hormone-dependent cancers exist [28,36]. On the other hand, the existence of hormones having antitumor activities has also been reported. For example, the pineal hormone, melatonin, has been known to have inhibitory actions on the induction and growth of cancers [3]. The pineal gland and the adrenal medulla are amine-secreting endocrine glands and related to each other in the embryogenesis (neuroectodermal origin) and the biochemical nature [33]. Melatonin is synthesized by hydroxyindole O-methyltransferase (HIOMT) and a lipid-soluble amine hormone of which nuclear receptors have been known to exist [3]. On the other hand, epinephrine is synthesized by another methyltransferase enzyme, phenylethanolamine N-methyltransferase (PNMT), and epinephrine and norepinephrine are water-soluble amine hormones of which information is transmitted by intracellular second messengers via receptors on the plasma membrane [1]. The adrenal medulla consists of a part of the sympatho-adrenomedullary system and the pineal gland is innervated with sympathetic, postganglionic nerve fibers from the superior cervical ganglia. Structures and functions of the adrenal medulla are influenced by experimental manipulations such as pinealectomy or melatonin administration [22].

Although functional relationships between the pineal gland and adrenal medulla and the antitumor activity of pineal hormone have repeatedly been shown, no reports have appeared with reference to the antitumor activity of adrenomedullary hormone. It has been well established that methylcholanthrene (MC) injected into the subcutaneous tissue induces fibrosarcoma in mice and rats [13,26]. From these, we previously showed that at 90 days after the MC injection, the incidence of palpable tumor (fibrosarcoma) was 1) highest in the pinealectomy (PX) + adrenomedullectomy (AMX) group, 2) second highest in the AMX group, 3) lower in the PX group, and 4) lowest in the normal group and that at 165 days after MC injection, the incidence of large tumors (≥ 1 g) tended to be higher in both the PX + AMX group and the AMX group than in other groups [22,23].

In the present study, we sought to confirm our previous results and investigate further the effects of adrenomedullary autotransplantation and injection of epinephrine on the tumor incidence. Preliminary report on this subject have been presented at the Japanese meetings of Anatomists [48,47].

Materials & Methods

A total of 154 male Wistar rats purchased from commercial source (Japan Charles River Company) at 21 days of age were used. Rats were kept two to three per cage and maintained in the windowless animal room under 24 hr light-dark (LD 12:12) cycle and at constant room temperature ($22 \pm 2^\circ\text{C}$). A part of rats were maintained at $24 \pm 2^\circ\text{C}$. Food (commercial diet

pellets: MF, Oriental Bioservice, Japan) and water were given *ad libitum*. At 28 days of age, animals were divided into four experimental groups, i.e. 1) control group (non-operated or sham-operated), 2) bilateral adrenomedullectomy (Bil. AMX) group, 3) right adrenomedullectomy + left adrenomedullary autotransplantation (AMX + AMT) group, 4) bilateral adrenomedullectomy + epinephrine injection (Bil. AMX + E) group, and surgeries were performed.

AMX was done by autotransplantation of the adrenal gland. For autotransplantation of the adrenal gland, under halothane anesthesia dorsal mid, or para-sagittal, incision was made and then bilateral retroperitoneal incisions were made, and then both right and left adrenal glands were extirpated. Fat and connective tissues surrounding the adrenal gland were removed. After cutting the renal capsule, whole adrenal gland with the capsule was inserted under the renal capsule. After the kidneys were returned to the original place, the retroperitoneum and the dorsal skin were sutured with a string (5-0 nylon or 4-0 silk). For one week after the surgery, 1% saline was given to the rats.

For AMT, the kidney was reached via a similar approach under the halothane anesthesia, and the left adrenal gland was extirpated and kept in the Hanks' solution. After the removal of cortical tissue using a sharp knife or forceps for microsurgery, the adrenomedullary tissue was inserted under the renal capsule. On the right side, the whole adrenal gland was transplanted under the renal capsule for AMX. For sham-surgery, retroperitoneal incision was made via the approach similar to the adrenal autotransplantation, and then hemiadenectomy was done or closed without adrenalectomy nor disturbance.

Histological changes in both transplanted whole adrenal tissue and adrenomedullary tissue were additionally examined in the Bil. AMX group and in the AMX+AMT group. Two rats were killed at each of 2, 4, 9, 14 and 28 days after transplantation in each group. From another rat of the AMX+AMT group, the autotransplanted adrenal medulla was taken out at 10 months after transplantation. Transplanted tissues were fixed in Bouin's fluid or Zamboni's fluid, and then embedded in paraffin. After serial sections were made at $7 \mu\text{m}$ in thickness, the sections were stained with hematoxylin-eosin. For each one of adrenomedullary tissues at 28 days and 10 months after transplantation, the immunohistochemical staining by immunogold-silver method was done using antibody for phenylethanolamine N-methyltransferase (PNMT) which is the epinephrine-synthesizing enzyme. At different time intervals after AMT, the volume of transplanted adrenomedullary tissue was measured using an image analyzer (IBAS, VIDAS, Karl Zeiss).

In the main experiment, about 2 mg of MC (or methylcholanthrene) crystals mixed with a small amount of cholesterol was inserted under the dorsal skin of the rats in the Bil. AMX group, AMX+AMT group and control group using a transplantation needle (Natsume Seisakusyo Company Ltd, Tokyo),

in most cases at four weeks, and in some cases at 6 weeks, after surgery. Tumor was examined by palpation at 90 days after MC insertion. After the palpation, rats were killed and transplanted tissues and tumors were taken out. Then the size of tumors were measured and the tissues of tumors and transplanted adrenals were examined histologically. After animals were used in the first half of the experiment at 90 days, 15 rats in each of the Bil. AMX group and the AMX+AMT group and 24 rats in the control group were kept raising until they were killed at 120 days or 165 days after the MC treatment. After tumors were examined by palpation, transplanted tissues and tumor tissues were examined similarly as those killed at 90 days. Epinephrine (600~800 ng/kg) was injected subcutaneously to 28 rats of the Bil. AMX group at 17:00~18:00 twice a week consecutively, starting from the next day of the MC treatment. If the mass of palpable tumor-like tissue was half a rice-grain in size, it was considered to be tumor tissue. Transplanted tissues were fixed with Bouin's fluid and tumor tissues were fixed with 10% formalin solution. Transplanted tissues were embedded in paraffin, and stained with hematoxylin-eosin after serial sections were made. After the size of tumors was measured, the tumor tissues were confirmed by histological examination. Experiments were repeated twice or more.

Statistical differences of the tumor incidence between experimental groups were examined by χ^2 -test, and as for tumor weight, Student's t-test was used.

All animal experiments in this paper followed the *Guidelines for Animal Experimentation, Hirosaki University*.

Results

Autotransplantation of the whole adrenal gland and the adrenal medulla

In the adrenal gland autotransplanted under the renal capsule, the whole adrenomedullary tissue and almost all the adrenocortical tissue became necrotic at two days after transplantation, and capsular cells and only a part of the glomerular zone remained. At 4 days after the transplantation, cortical cells showed active mitotic activities. Mitotic figures were more frequently seen in the glomerular zone. At 14 days, the fascicular zone was distinguishable. At 28 days, the reticular zone became distinguishable and the three cortical zones were almost accomplished (Fig. 1). Adrenomedullary cells were not seen in all cases and at all days examined after the transplantation.

In the autotransplanted adrenomedullary tissues, no mitotic figures were seen throughout the post-surgical duration. The volume value of transplanted adrenomedullary tissue was under 0.291 mm^3 , which was under a half of the volume of hemilateral adrenal medulla in control rats at 21 weeks of age, and showed a large variation. In the long-term cases of autotransplantation, i.e. more than 118 days after

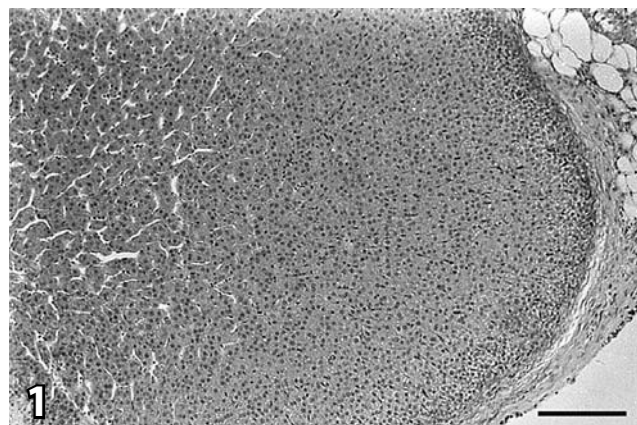


Fig. 1. Light micrograph of regenerated adrenal cortex at 28 days after autotransplantation of the whole adrenal gland. H-E. Bar:100 μm

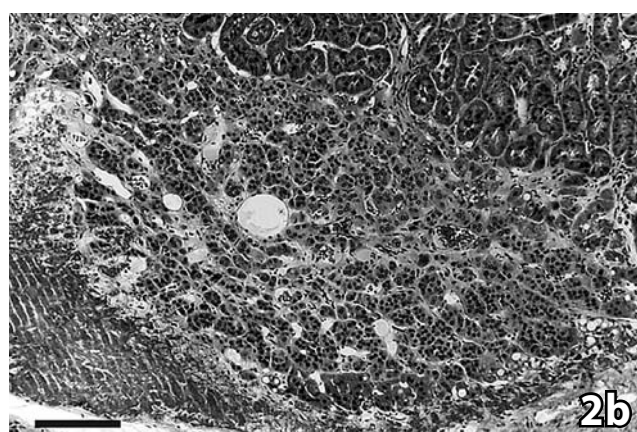
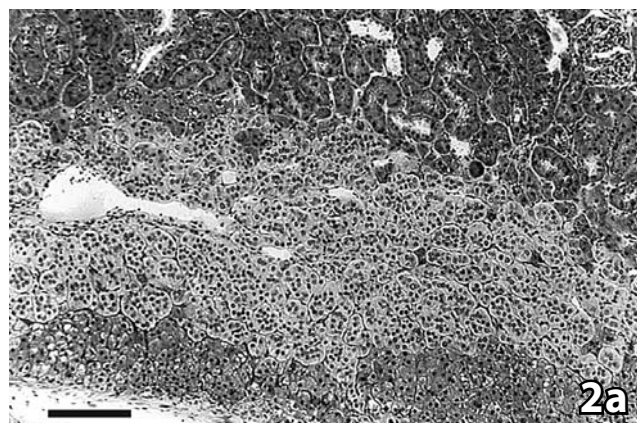


Fig. 2. Light micrographs of transplanted adrenomedullary tissues at 130 days after transplantation. H-E. Bar:100 μm
 a: This tissue shows similar appearance to normal adrenomedullary gland.
 b: This tissue shows slightly looser structure and is composed of relatively small parenchymal cells.

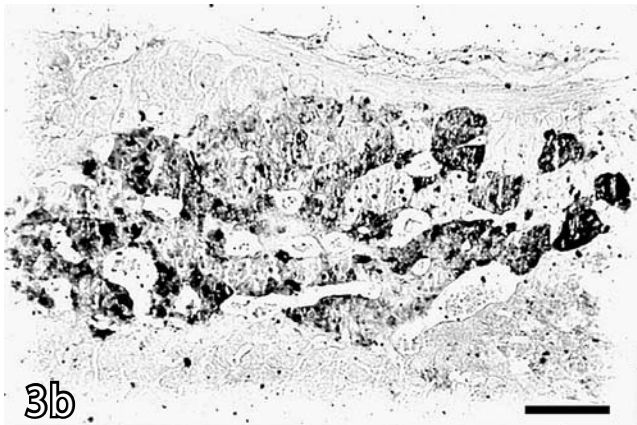
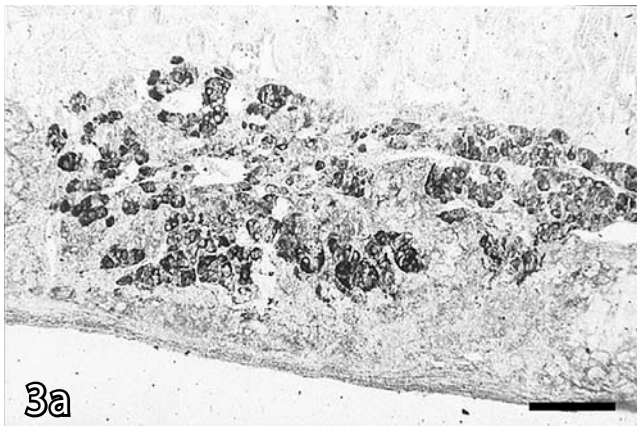


Fig. 3. PNMT-immunoreactivity of the autotransplanted adrenomedullary glands. Immunogold-silver method. Bar:100µm
a: The reactivity is positive at 28 days after transplantation.
b: The reactivity is still positive at 10 months after transplantation

surgery, various types of histological figures were seen. The transplanted tissues showed 1) histological figures almost similar to normal ones (Fig. 2a), 2) necrotic figures in a part of the adrenomedullary tissue, and 3) a loose structure as a whole which was composed of relatively small-sized cells (Fig. 2b). The ratio of cases, in which the transplanted adrenomedullary tissue was alive, was 100% in a total of 10 cases examined until 28 days after transplantation, 95% in 21 cases from 118 to 130 days after transplantation (90 days after MC treatment), and 66.7% in 15 cases at 193 days after transplantation (165 days after MC treatment). PNMT immuno-reactivity was positive in a large area of each autotransplanted adrenomedullary tissue at 28 days and 10 months after transplantation (Fig. 3).

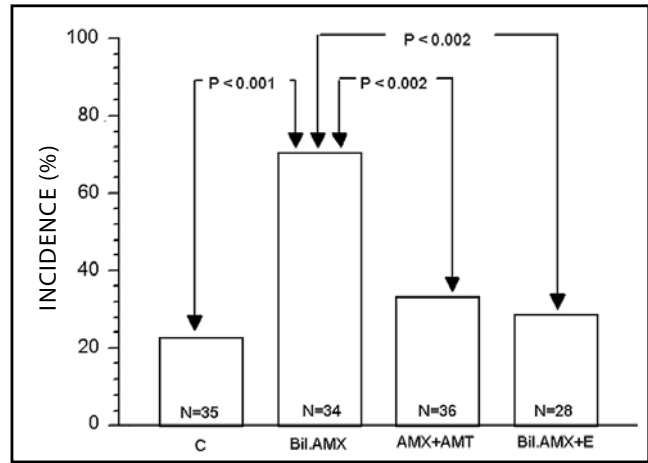


Fig. 4. The incidence of tumor at 90 days after methylcholanthrene insertion in different groups. C: control

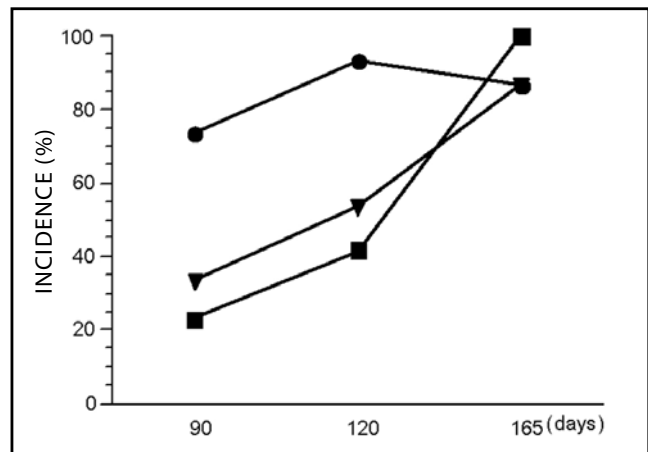


Fig. 5. Changes in the incidence of tumor after methylcholanthrene insertion. ■: Control group; ●: Bil. AMX group; ▼: AMX + AMT group

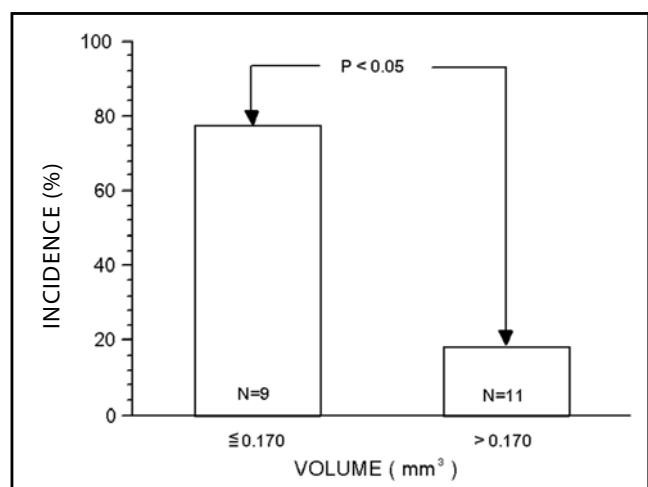


Fig. 6. The relationship between the volume of transplanted adrenomedullary tissue and the incidence of tumor

Methylcholanthrene-induced fibrosarcoma

The incidence of rats having palpable tumor in the subcutaneous tissue on the back was 8/35 (22.9%) in the control group and 24/34 (70.6%) in the Bil. AMX group at 90 days after MC treatment. In contrast, the tumor incidence showed lower values, i.e. 12/36 (33.3%) in the AMX+AMT group ($P<0.002$) and 8/28 (28.6%) in the Bil. AMX+E group ($P<0.002$) (Fig. 4). At 120 days after MC, the frequency was 14/15 (93.3%) in the Bil. AMX group and 8/15 (53.3%) in the AMX+AMT group which was significantly lower than the former value ($P<0.02$). But at 165 days after MC, the incidence of tumors visible by autopsy in the AMX+AMT group showed a high value, 12/15 (80.0%), which was not significantly different compared with that of the Bil. AMX group, 13/15 (86.7%), and the incidence became 100%, even in the control group (24/24) (Fig. 5). The mean weight of tumors at 165 days after MC was heavier in the Bil. AMX group (mean \pm S.E: 25.9 \pm 23.3g) than in the AMX+AMT group (15.5 \pm 10.7g), but no significant difference was seen between those in the two groups.

In the latter part of the experiment, when rats were sacrificed and examined at 90 days after MC, the incidence of tumor was 13/19 (68.4%) in the Bil. AMX group and 9/21 (42.9%) in the AMX+AMT group, and the latter value tended to be lower ($P<0.11$) but was not different significantly. The weight of tumors was not different also between the two groups (Bil. AMX group, 40.9 \pm 23.3mg; AMX+AMT group, 44.4 \pm 18.7mg). However, when the tumor incidence was compared between the transplanted tissue-not-well-preserved group (the tissue volume ≤ 0.17 mm³) and the tissue-well-preserved group (the volume >0.17 mm³), the tumor incidence in the former, 7/9, was significantly higher than that in the latter, 2/11, ($P<0.05$) (Fig. 6). When the transplanted adrenomedullary tissue contained relatively large necrotic tissue, the volume of alive cortical tissue tended to be smaller. And the larger the preserved medullary tissue was, the larger the preserved cortical tissue tended to be.

In most cases of tumor-like tissues, spindle-shaped cells having rounded nuclei (mixed with large and small ones) and irregularly running collagen fibers were seen, and sometimes infiltration with cells such as small lymphocytes was seen also. The cases showing these histological characteristics were considered to be tumor (fibrosarcoma). The tumor incidence confirmed by autopsy was almost similar to that checked by palpation.

Discussion

Autotransplantation of the adrenal gland

It has long been known that after autotransplantation of the whole adrenal gland, most of the transplanted tissue becomes necrotic, and then new cortical tissue is reconstructed by regeneration, except the adrenomedullary tissue. According to Srougi and Gittes [42], when the whole adrenal gland or small

pieces of the adrenal gland are autotransplanted under the renal capsule, most of the transplanted tissue becomes necrotic and then cells from the glomerular zone to the reticular zone regenerate by 3~4 weeks after transplantation. The plasma glucocorticoid level recovers to the normal level by 7~8 weeks after transplantation. However, although the whole amount of steroids in the cortex is retained, the relative amount of different steroids changes [11] and the plasma steroid level after stress is lower than the normal level. Wilkinson et al. [44] and Engeland [14] reported that the plasma corticosterone level secreted from the regenerated tissue recovers to normal level from the lowered level during the regenerative period, but the plasma ACTH level is higher than the control level and the corticosterone responsiveness to ACTH is decreased in the regenerative tissue.

In our experiment of the whole gland transplantation, the process of necrosis and regeneration after autotransplantation was similar to the results described by Srougi and Gittes [42], and absolutely no alive tissue of the adrenal medulla was seen in all cases examined. Therefore, medullectomized adrenal glands can be made by this method and the plasma glucocorticoid level is considered to recover to nearly normal level gradually after a long period of regeneration and hypofunction of the autotransplants.

It is well-known that the high concentration of glucocorticoid secreted from the adrenal cortex plays an important role for the epinephrine synthesis [1]. However, it has been reported that when the adrenomedullary tissue is transplanted into the CNS, hormonal substances are synthesized and secreted from the transplanted tissue to a certain extent. For example, Freed et al. [16] reported that epinephrine is contained in adrenomedullary cells even at 5 months after transplantation into the brain and the contents are variable from patient to patient. Sagen et al. [34,35] reported many cases of allograft of adrenomedullary tissue into the subarachnoid space. According to these reports, transplanted adrenomedullary cells survive for at least 4 months and keep the ability of synthesis and secretion of epinephrine and enkephalin to a certain extent. In the present experiment also, the PNMT-like immunoreactivity was seen at 28 days and 10 months after transplantation, although the volume of transplanted adrenal medulla was smaller than that of normal medulla. From these, it would be reasonable to speculate that a certain amount of epinephrine is still synthesized and secreted from the transplanted adrenomedullary tissue in the present experiment.

On the other hand, it is likely that a fairly large variation exists in the functional activities of transplanted medullary tissue and the amount of hormones secreted at 90~120 days after the MC injection, as shown in the results of our present study and in those of Freed et al. [16]. This is probably because the amount of cortical cells mixed within the transplanted medullary tissue and conditions of adrenomedullary graft are variable at the time of transplan-

tation and thereafter, and in a part of the animals, the duration between the AMT and the MC injection was about two weeks longer. As shown in Fig. 6, it seems possible that these differences in the amount of well-preserved, i.e. alive and active, adrenomedullary tissue cause variations in the amount of hormones secreted and the functional activity including the possible tumor-suppressing activity as discussed below.

Tumor-suppressing effects of adrenomedullary autotransplantation and epinephrine

It has been well established that epinephrine occupies the highest amount in catecholamines secreted from the adrenal medulla, from which the plasma epinephrine mostly derives. In contrast, only a small amount of norepinephrine derives from the adrenal medulla, and the most part of plasma norepinephrine is released from postganglionic sympathetic nerve endings in the whole body. The plasma norepinephrine level is generally unchanged after AMX, but the free epinephrine level falls to essentially zero [17]. Our present results clearly show that the induction and growth of fibrosarcoma by MC is stimulated by AMX and inhibited by adrenomedullary hormones, most probably epinephrine. The tumor-stimulating effect of AMX was apparent in the early period, but not clear at 165 days after carcinogen insertion. Even at 165 days, however, the mean value of tumor weight was higher in the AMX group than in the control and AMX+AMT groups. This result seems to reflect our previous observation that large tumors ($\geq 1g$) occur more frequently in the AMX group than in the control and AMX+AMT groups at 165 days after MC treatment [22].

It is now widely accepted that cancer is the result of a multi-step process [28,26]. Carcinogenesis consists of a first phase of initiation followed by various phases of promotion. With a few notable exceptions, hormones have been thought to act as promoters or cocarcinogens which by themselves are nontumorigenic. Therefore, as the action sites of protective mechanisms of host against cancer, there are various possibilities of direct actions on not only a first step of initiation but also subsequent promotion-related steps. In addition, the possibilities of indirect actions such as blood supply and immunological mechanisms also exist.

It has been known that a marked difference between epinephrine and norepinephrine exists in the action via the β_2 -receptor, but not in those via other receptor types. In the present study, since norepinephrine remaining in the blood of AMX rats was ineffective in the tumor-suppressing effect, it is likely that epinephrine plays such an antitumor role via the β_2 -receptor. The β_2 -receptor-mediated action of epinephrine includes various activities such as glucose-mobilization from liver cells and increased secretion of ACTH from anterior pituitary cells [1]. Thus, epinephrine causes a hyperglycemic state. On the other hand, under the present experimental situation, AMX probably causes a hypoglycemic state by both the

decreased secretion of glucocorticoids [44,14] and the depletion of epinephrine.

According to Mainwaring (1986) [28], growth hormone (GH) has been considered a good example of promoter in the induction of skin tumors in mice by MC and croton oil. GH acts as a promoter by at least two mechanisms: 1) increasing uptake of glucose and amino acids, and 2) 'sensitization', i.e. by this process, powerful mitogen, insulin-like growth factor-I (IGF-I)(induced and/or stimulated by GH), increases the potential number of cells that will be at risk on exposure to initiating agents. It has been known that hypoglycemia stimulates the release of GH, whereas hyperglycemia inhibits GH secretion, probably via the hypothalamus [7,19]. Therefore, it is possible that a hypoglycemic state due to AMX stimulates the induction and growth of tumor at least partly by changing the influence of promoter, i.e. GH and/or IGF-I, and AMT or epinephrine causes tumor suppression by reversing such a hypoglycemic state.

In addition, there is another possibility that the blood glucose level influences the tumor growth via the blood supply. Owing to the recent research progress on vascular neoplasia in various diseases including cancer, new tissue factors and pathophysiological mechanisms emerged [49,8]. Vascular endothelial growth factor (VEGF) is a typical one of such and has been reported to be secreted in response to hypoglycemia by many researchers [40,12]. In the present study, therefore, the following mechanisms can be tentatively thought: 1) hypoglycemia occurred in AMX rats induces the increased expression of VEGF, which in turn stimulates vascular neoplasia in the tumor, and 2) epinephrine injected or secreted from transplanted adrenomedullary cells reverses the hypoglycemia and suppresses the VEGF expression and vascular neoplasia in the tumor.

It is also possible that epinephrine (AMX) causes the increased (decreased) cAMP level in precancer fibroblasts [31,2], which in turn causes arrest (activation) of tumor induction and growth [30,37]. Relating to this, it is interesting to note that epinephrine has been reported to inhibit the invasion of cancer cells by increasing intracellular cAMP [46]. On the other hand, recent data demonstrating that genomic hypomethylation causes chromosomal instability and induces tumors in mice strongly suggest that epigenetic changes may directly contribute to tumor development [26,15]. Since DNA, as well as epinephrine and melatonin, is methylated by methyltransferase enzymes mainly using S-adenosylmethionine as the methyl donor [32], there may be some pathophysiological relationship between tumor promotion and these two amine-secreting glands. But more experimental data are needed to elucidate such a possibility.

At the present time, it is not apparent whether the actions of epinephrine on immunological mechanisms [10,27] contribute to the antitumor activity of the adrenal medulla, since supporting evidence is less clear and even opposite results have been reported [43,41].

In addition to catecholamines, various biologically active substances are also contained in the adrenal medulla, e.g. met- and leu-enkephalins [38,6,45] and interleukin-1 [39]. The amount of met-enkephalin is highest among various peptides [45] and most of the circulating met-enkephalin is derived from the adrenal medulla [17]. The met-enkephalin synthesis is increased by denervation [38]. Many experimental data from both in vivo and in vitro studies have shown that met-enkephalin is immunostimulant which activates T-lymphocytes and NK cells and enhances mitogen-induced proliferation of lymphocytes [5,9,4,29]. Therefore, the antitumor activity of adrenal transplant observed in this study may be, at least in part, due to immunostimulatory effect of met-enkephalin and interleukin-1. However, although an inhibitory autocrine/paracrine action of met-enkephalin on adrenomedullary catecholamine release [25,20] has been widely accepted, systemic effect of enkephalins via circulation remains questionable at present [17], mainly because enkephalins are small peptides and rapidly deactivated and their plasma levels are low [38,18]. Since a small number of lymphocytes have been observed electron microscopically in the vicinity of rat adrenomedullary chromaffin cells by ourselves [21] and also in bovine adrenomedullary chromaffin cell cultures by Kovals et al. [24], the possibility of interplay among neuro-immuno-endocrine mechanisms [5] in the adrenal medulla still remains.

In conclusion, it is possible that epinephrine inhibits the induction and growth of fibrosarcoma by MC by various mechanisms including actions on the promotion process involving GH secreted from anterior pituitary cells and cAMP-related chemical events in fibrosarcoma cells (or precancer fibroblasts) and on the blood supply for cancer (or precancer) tissue by vascular neoplasia. However, more experiments are needed to elucidate to what extent and how medullary catecholamines and/or peptides actually contribute to immunological protection against tumor via paracrine effects and/or systemic effects through circulation, and to obtain a definite conclusion on the mechanism of possible antitumor activity of the adrenal medulla.

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