Experimental Radiation Therapy and Apparent Radioresistance of Autochthonous Tumors Subcutaneously Induced with 3-Methylcholanthrene in Mice

Hiroshi Tanooka, Hiroshi Hoshino, Kazuhiko Tanaka, and Mizue Nagase

ABSTRACT

The radiation response of autochthonous tumors induced by s.c. injection of 3-methylcholanthrene in ICR/JCL mice was studied. The tumors were mostly fibrosarcomas and grew with an average volume-doubling time of 2.6 days, independently of the time of tumor appearance or dose of the chemical used. The tumors were locally and singly irradiated with 6-MV X-rays through a filter. Autochthonous tumors were similar to their transplants in postirradiation regression and gross cellular radiosensitivity ($D_0$, 400 rads), as estimated from regrowth time. However, most of the autochthonous tumors irradiated with single doses of up to 7.4 kilorads recurred within 120 days, while in identically irradiated tumors transplants a complete cure was obtained with doses of 6.5 kilorads or more. The 50% tumor control dose with no recurrence within 120 days was 4.4 kilorads for the transplants. Transplantation of 26 irradiated autochthonous fibrosarcomas produced only a few tumors in autochthonous hosts, and they were completely rejected in others, previously tumor-free mice; a 48% recurrence was noted even after resection of the irradiated tumor. Two possibilities for the apparent radioresistance of autochthonous tumors were suggested: (a) existence of radioresistant cells in autochthonous tumors; and (b) induction of a second new tumor by the additive effect of the chemical and radiation. Although our results are preliminary, the second possibility is not favored since autochthonous tumors induced with low doses of 3-methylcholanthrene recurred with high frequency after irradiation. Moreover, irradiation of tumor-free mouse skin that had been treated with the carcinogen produced tumors at a very low frequency.

INTRODUCTION

Most investigations on experimental radiation therapy have been with tumors transplanted to experimental animals. These tumors have been shown to be completely curable by single exposure to ionizing radiation. The radiation dose yielding a TCD$_{50}$ without recurrence within a certain postirradiation period appears to be a suitable indicator of the efficacy of the radiation therapy applied (11-13).

Kallman and Tapley (9) used spontaneous mouse mammary tumors as a model for experimental radiation therapy of autochthonous tumors and obtained TCD$_{50}$ (100 days) of 4.23 kilorads with single X-irradiation. Suit and Shalek (12) obtained 6.20 kilorads for TCD$_{50}$ (170 days) with this type of tumor under local anesthesia. However, spontaneous mammary tumors may be more responsive to radiation than are chemically induced tumors. Tokuzen et al. (14-17) applied immunotherapy to both spontaneous and chemically induced tumors in mice and obtained little response as compared with the case of transplanted tumors. However, to our knowledge, no investigation has been reported concerning the radioresponse of chemically induced autochthonous tumors.

It has been postulated that most human cancers are caused by environmental chemical carcinogens (3). In the present study, we attempted to control chemically induced autochthonous tumors by X-irradiation and compared the results with those obtained with identically treated transplanted tumors. The observed difficulty in controlling the chemically induced autochthonous tumors may be related to the mechanism(s) of carcinogenesis at the autochthonous tumor origin.

MATERIALS AND METHODS

Mice. Female virgin ICR/JCL mice (Clea Japan, Tokyo, Japan), 6 weeks old, were used. All mice had access to a mouse diet (CE-1; Clea Japan) and water ad libitum and were kept in an isolating rack.

Production of Autochthonous Tumors. Tumors were induced with MC (Fluka AG, Buchs, Switzerland) as described by Tokuzen et al. (17). A solution of MC (0.5, 2.5, or 5 mg/ml of olive oil) was prepared by autoclaving, and 0.1 ml of this solution was injected s.c. into the right groin of the mice. Approximately 30 mice/group were independently treated. Tumor size was measured with calipers, and tumor volume was estimated according to the formula $4/3\pi ab^2$, where $a$ and $b$ are the halves of the long and short axes of the tumor. Tumor volume-doubling time was determined from the initial slope of the growth curve. Mice were autopsied, and tumor types were histologically determined.

Tumor Transplants. MC-induced tumors were transplanted to the right groin of ICR/JCL mice by s.c. injection of a solid piece of the tumor, using a transplantation needle, and serial tumor transplantations were performed. The tumor type was histologically confirmed as fibrosarcoma at each transplantation. The tumor take was almost 100% up to the third transplantation; thereafter, it dropped to 60 to 70% up to the ninth transplant. Second-generation tumors were irradiated with doses of 5.6 kilorads or more, and the fourth and eighth generations were used for 3.8- and 4.7-kilorad irradiations, respectively.

Irradiation. Single X-irradiation was performed when autoch-
thonous or transplanted tumors reached around 8 to 10 mm in diameter. The mice were anesthetized with an i.p. injection of 0.04 mg Nembutal (Abbott Laboratories, Chicago, Ill.) per g of body weight, and their legs were tied to pins on a cork board. Mouse trunks were covered by and fixed with a thin lead plate which was pegged to the board and left the tumor site exposed. X-rays were generated by a 6-MV medical linear accelerator (NELAC 1006; Nihon Electric Co., Tokyo, Japan) and filtered through a 1-cm-thick acrylic resin plate. The irradiation field was 3 x 3 cm square, which included the tumor near its corner. To avoid back-scattering of X-rays, the cork board was placed on a frame of polystyrene foam. The dose rate at 70 cm from the target was about 800 rads/min, and the total dose delivered was counted by a preset integrator which had been calibrated by an ionization chamber (Ixon, type 2500/3; Nuclear Enterprises Ltd., Edinburgh, U.K.).

Criterion for Cure Designation. Mice that showed complete tumor regression and survived for 120 postirradiation days without recurrence were considered to be cured.

RESULTS

Autochthonous Tumor Formation. The time course of tumor formation at the injection site of 3 different MC doses is shown in Chart 1. There was a correlation between tumor yield and dose of MC. The histogram in Chart 1 shows the rates of tumor formation every 20 days after treatment with 0.5 mg MC. The peak tumor formation rate appeared around the 100th postinjection day, and the final tumor yield obtained from 3 separate autochthonous tumor system to provide fairly homogeneous tumors. Their uniform volume-doubling times, we consider this autochthonous or transplanted tumors are not necessarily "slow growers." Their volume-doubling time is independent of either the MC dose or the time required for these tumors to regress to one-half of their original volume was 6.1 days. However, all irradiated tumors, with the exception of one cure, recurred (Table 1) and regrew with an average volume-doubling time of 2.7 days. Of 31 recurrent tumors, 28 were examined and all of these were fibrosarcomas, with the exception of one squamous cell carcinoma which recurred after 1.9 kilorads of irradiation. The radiosensitivity of gross tumor cells in situ was estimated from the tumor regrowth time according to the method of Suit and Shalek (12); the dose for one lethal hit ($D_0$) was approximately 400 rads.

To examine the possibility that the apparent radioresistance of the autochthonous tumors was due to new, second tumor formation, we irradiated these tumors with 6.5 kilorads and excised them immediately (Chart 3). The excision site was sutured (Site 1), and the tumor was cut into 2 larger pieces and one smaller piece. One of the larger pieces was transplanted to the neck of a paired tumor-free ICR mouse (Site 3). The remaining smaller piece was used for histological examination. These tumors included 26 fibrosarcomas, 2 mammary adenocarcinomas, and 1 squamous cell carcinoma. Tumor regrowth of Sites 1, 2, and 3 was then monitored for 120 days. Mice that died without apparent tumor regrowth within the 120-day observation period were not counted as effective and were excluded from the results of this experiment. After resection of irradiated fibrosarcomas, tumor development was noted at 48% of Site 1 (10 of 21) and 19% of Site 2 (4 of 21), and no tumor development was noted at Site 3 (0 of 25). All tumor regrowths were identified as fibrosarcomas. Of 20 effective pairs of mice, 9 pairs showed no tumor regrowth at any of the 3 sites.

One case of definite new tumor induction was found at Site 1, where fibrosarcoma developed after the resection of an irradiated squamous cell carcinoma. However, 239 days were required for its appearance. Mammary adenocarcinoma did not regrow at any of the 3 sites.

Radiation Response of Autochthonous Tumors Induced with the Lower Doses of MC. If the apparent radioresistance of MC-induced tumors is in fact due to new tumor formation, then it is reasonable to assume that tumors induced with the lower MC doses would show a lower rate of recurrence. Tumors produced with 0.25 or 0.05 mg MC were irradiated with 6.5 kilorads. As seen in Table 1, irradiated tumors recurred with considerable frequency, although some cures were found.

Radiation Response of Transplanted Tumors. Transplanted fibrosarcomas grew at an average volume-doubling time of 2.0 days. No spontaneous regression occurred among these transplants, and all hosts died within 80 days, by which time the tumor had reached a maximum size of 65 cu cm (Chart 2c). When the transplanted tumors were irradiated with doses of 6.5 kilorads (Chart 2d) or more, they regressed at an average time to return to one-half of their original volume of 4.7 days and were completely cured. At lower irradiation doses, the cure...
Radiation Response of Autochthonous Tumors

Chart 2. Effects of single X-irradiation on MC-induced autochthonous fibrosarcomas (a, b) and their second-generation transplants (c, d). Doses (in kilorads) given were: a, 0; b, 6.5; c, 0; d, 6.5. Tumor volumes were plotted against time after irradiation until death (○) or sacrifice (x) of mice. All recurring autochthonous tumors were fibrosarcomas. Hatched regions, nonpalpable tumors. Right ordinate, diameter of the tumors, assuming them to be spherical.

Table 1

<table>
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<tr>
<th>Tumor</th>
<th>Radiation dose (kilorads)</th>
<th>No. of mice irradiated</th>
<th>No. of mice at the first recurrence (A)</th>
<th>Recurrence within 120 days (B)</th>
<th>Av. tumor regrowth time* (days)</th>
<th>No. of cures at 120 days</th>
<th>Tumor cure rate [1 – (B/A)] (%)</th>
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* Average time required for irradiated tumor to regrow to the size before irradiation.
† One squamous cell carcinoma is included, but it is excluded in estimation of the average volume-doubling time.
‡ Tumor type is unknown.
§ Tumors were produced with 0.25 mg MC.
* Calculated by the “product limit” method of Howes (7). No change in other values.
* Tumors were produced with 0.05 mg MC.
† Number of mice on the 120th day after irradiation.

rate decreased (Table 1). All recurrent tumors were fibrosarcomas. TCD50 (120 days) for the transplanted fibrosarcomas was estimated to be 4.4 kilorads from probit plotting of the cure rate against the radiation dose. The D0, estimated from the tumor regrowth time at 3.8 kilorads of irradiation, was approximately 400 rads, similar to the case of autochthonous tumors.

Late Effects of Irradiation. In mice with transplanted fibrosarcomas which were either cured or not cured by X-irradiation, only one metastasis were detected. Cured mice which had received 5.6 to 7.4 kilorads died gradually without recurrence of cancer; 55% of these survived until the 300th postirradiation day, while 95% of nonirradiated normal mice maintained under identical conditions survived for this period. In 28 cured mice, 2 papillomas, one mammary adenocarcinoma, one lymphoma, one leukemia, and one fibrosarcoma developed during the 400-day observation period. The fibrosarcoma developed at the site of the 7.4-kilorad irradiation after 397 days, accompanying metastasis to the lymph node.

In the group of 31 mice in which autochthonous fibrosarco-
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was resected in addition to the 6.5-kilorad irradiation accom-
panying metastasis to the chest, lung, stomach, spleen, and
abdomen, respectively.

Test of Tumor Induction in MC-treated Skin with X-Rays.
Tumor-free mice were selected among animals that had been
treated with 3 doses of MC and maintained for 250 days. The
MC injection site was irradiated with 6.5 kilorads to test the
additive tumorigenic effect of MC and X-rays. Of 5 mice treated
with 0.5 mg MC, one developed fibrosarcoma which appeared
at the irradiated site 70 days after irradiation. Of 6 mice treated
with 0.25 mg MC and 19 mice treated with 0.05 mg MC, none
developed tumor during 120 days (1 animal died in the former
group; 6 animals died in the latter group).

DISCUSSION

Difficulties in autochthonous tumor experiments, especially
in the limited availability and heterogeneity of these tumors,
have been pointed out by Kallman (8). In the present autoch-
thonous tumor system, tumor yield is high, tumors are pro-
duced in a relatively short period (peak, 100 days after 0.5 mg
MC injection; see Chart 1), and the tumor type is mostly
fibrosarcoma. As judged from volume-doubling time, tumors
grow with a constant speed, independent of the time of ap-
pearance and carcinogen dose. Therefore, we consider these
tumors to be homogeneous.

Assuming a constant volume-doubling time of 2.6 days, it
takes 60 days for a single tumor cell (volume, $1.7 \times 10^{-9} \text{ cu cm}$) to reach a palpable size ($1.4 \times 10^{-2} \text{ cu cm}, 3 \text{ mm}
diameter). This supports use of the TCD$_{50}$ (120 days) as a valid
criterion to assess tumor control, since a single tumor cell
surviving radiation is capable of producing a palpable tumor
within 120 days on the assumption of immediate start of growth
after irradiation with a constant growth rate.

Furthermore, the present mouse system entails a lower ra-
diation-induced tumor background. Using either single irradi-
ation with 3 kilorads of $\beta$-rays from $^{90}$Sr-$^{90}$Y (6), which is an
optimum tumor production dose in rat skin (2), or repeated
irradiation with the same dose, almost no tumors developed in
the skin of the present mouse strain. This low background is
favorable in long-term tests for recurrence of irradiated tumors.

It is most striking in the present results that most of the
irradiated autochthonous fibrosarcomas recurred after tempo-
rary regression even with highest possible dose of radiation.
This recurrence was not due simply to failure of the irradiation
to kill many, perhaps most, tumor cells, since identically irra-
diated transplanted tumors were cured. The curability of trans-
planted tumors was confirmed also by using inbred mice (WB
$\times$ C57BL/6J F₁), tumors transplanted from a 0.5-mg MC-
induced primary tumor in these mice exhibited no recurrence
after 6.5 kilorad irradiation (data not shown). In many aspects,
the autochthonous fibrosarcomas resembled their transplants:
volume-doubling time (2.6 versus 2.0 days); post-irradiation
regression time (6.1 versus 4.7 days); volume-doubling time at
recurrence (2.7 versus 2.7 days); and gross cellular radiosen-
sitivity estimated from tumor regrowth time ($D₀$, 400 rads).
Thus, the apparent resistance of autochthonous tumors cannot
be explained by known radiobiological mechanisms such as
cellular radioresistance due to low oxygen tension in tumors.

Therefore, we considered 2 possible mechanisms for the
apparent radioresistance of autochthonous tumors: (a) the
existence of a small radioresistant tumor cell fraction in au-
tochthonous tumors; and (b) induction of a new second tumor
by irradiation. Concerning the first possibility, such high radi-
oresistance is possible for cultured human melanoma and HeLa
cells depending on cloning conditions ($D₀$, 4300 rads in agar,
190 rads in plate for HeLa) according to the report of Good et
al. (5). The high rate of metastasis found after resection of 6.5-
kilorad-irradiated autochthonous fibrosarcomas indicates the
presence of tumor cells surviving irradiation. If such radiore-
sistant cells survive in the autochthonous tumor mass, then it
is reasonable to posit that transplants of irradiated autochtho-
nous tumors may develop a tumor at the transplantation site.
However, the transplants of tumors irradiated with 6.5 kilorads
developed a tumor at the nonirradiated transplantation site
of the same mouse with only a 19% frequency (Chart 3, Site 2).
Furthermore, no tumor development was noted at Site 3. While
it appears that only a few cells survived irradiation, the possi-
bility cannot be ruled out that surviving tumor cells were re-
jected by some kind of host-mediated reaction at the trans-
plantation site even in the same host and were capable of
flourishing only in situ.

The considerable recurrence that was found even after re-
section of the irradiated tumor (Site 1) may appear to support
the second suggestion and what we considered to be "recur-
rent" tumors may in fact represent new second tumors that
developed at the site of the first, carcinogen-induced tumor.

* H. Tanooka and H. Hoshino, unpublished observations.
However, an optimum surface dose for the induction of adnexal tumors in rat skin is about 3 kilorads; at higher doses, the tumor yield becomes lower if radiation alone is used for tumor induction (2). With doses ranging from 1690 to 4000 rads, squamous cell carcinomas are induced with a very low frequency in both rats and mice, 0.026 squamous cell tumor per sq cm in mouse skin (1). Compared with these carcinogenic radiation doses, our tumor control experiment doses (e.g., 6.5 kilorads) were much higher. Furthermore, for s.c. tumor induction with X-rays, the frequency is thought to be much lower. On the other hand, the additive effect of MC and X-rays must be considered. In rats, the additive effect of i.g. administration of 40 mg MC and 400 rads whole-body X-irradiation on mammary tumor induction has been reported (10). A similar additive effect has been observed in leukemia induction in mice treated by MC painting and 175 R X-irradiation (4). However, the low tumor incidence noted after irradiation of mice that had developed no tumor after MC treatment and the high frequency of post-irradiation recurrence of autochthonous tumors produced with low doses of MC (0.25 and 0.05 mg) make new tumor formation an unlikely possibility. However, since neither of the 2 possibilities that we considered is fully substantiated and since our technique in the present experiments entails some limitations, further studies are necessary before a conclusion can be made.

It is generally recognized that chemical carcinogens, naturally occurring as well as artificial, are involved in the cause of most human cancers. To advance the treatment of human cancers, the development of an experimental system with chemically induced autochthonous tumors is of vital importance.

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REFERENCES


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