The Specific Induction of Osteosarcomas in Different Mouse Strains after Injections of $^{239}$Pu Citrate

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**Carcinogenicity/Bone tumors/Soluble $^{239}$Pu/Strain differences/Mouse.**

Lifetime bone tumor induction by the injection of a bone-seeking alpha emitter, $^{239}$Pu citrate, was compared among 630 female mice from three strains (C3H/He, C57BL/6 and B6C3F1) showing different genetic backgrounds for carcinogenesis. Bone tumors, mostly osteosarcomas, appeared early during the period from 200 to 600 days after the injection, showing an almost similar dose responsiveness with a peak incidence of 50% to 63% at skeletal doses of 2–3 Gy, in all mouse strains. The primary sites of bone tumors from these strains were also predominantly distributed in 80% to 90% of the skeletal bones, which had well-developed trabecular bone surfaces and large vascular sinusoids. The frequency of lymphoid neoplasms was significantly lower than the control values, and some appeared earlier at the higher injected doses than those of the controls. Fewer or no myeloid leukemias were found in all the control and injected animals, and the incidences of other solid tumors decreased, reaching zero at doses where the maximum incidences of bone tumors were noted. These findings indicate that osteosarcoma is the only specific tumor commonly observed among different mouse strains following the injection of soluble plutonium compounds.

**INTRODUCTION**

It has been well recognized that bone-seeking and alpha-emitting radionuclides preferentially induce bone tumors in the human cohorts of radium ($^{226}$Ra) dial painters, radium ($^{224}$Ra)-injected ankylosing spondylitis patients, and recently in Mayak workers who have been exposed to plutonium ($^{239,238}$Pu) compounds. However, limited information or statistical uncertainty from these epidemiological studies does not fully permit estimates of risk factors and toxicity for bone cancer induction by alpha emitters. Experimental studies on bone carcinogenesis by injection of plutonium and other alpha emitters have been fully described in mice, rats, and dogs, implying that the toxicity ratio of $^{239}$Pu in comparison to $^{226}$Ra for bone carcinogenesis is the highest (approximately 15 to 16) among bone-seeking alpha emitters, irrespective of animal species. Supposing that the toxicity ratios are common between animals and humans, the risk factor for bone cancers from plutonium is estimated to be 1,200 in humans vs. 12,000 in dogs per 10⁶ rad as described. Nevertheless, it has not been elucidated whether only bone tumors are specific to alpha-emitting bone-seekers translocated through blood into the skeletal bones, and whether such a tumor spectrum is common among animal species and strains with different genetic backgrounds for spontaneous and radiation carcinogenesis. Our previous studies showed a relatively higher frequency of lymphoid neoplasms in C3H/He mice at higher skeletal doses over 10 Gy after injections of soluble $^{239}$Pu citrate, whereas both osteosarcomas and lymphomas were differentially induced by $^{239}$Pu-injection in C3H/He, C57BL/6, and B6C3F1 mice, which respectively show different tumor spectra before and after external radiation exposures.

In consideration of these findings, the present study was done to clarify the specific carcinogenicity of injected soluble $^{239}$Pu citrate to induce osteosarcomas by comparing the differences in lifespan carcinogenesis among three strains of mice. The results are discussed in comparison to other alpha emitters or radiations and as focused on the sensitivity and related mechanisms for bone tumorigenesis.

**MATERIALS AND METHODS**

**Experimental animals**

Specific pathogen-free female mice of three strains, C3H/HeN (C3H), C57BL/6J (C57), and their hybrid B6C3F1 (BC3), were purchased from a breeding facility (Japan SLC Co.), and all the animals were housed 10 per polycarbonate cage, given a commercial diet (Funabashi Farm Co.) with water ad libitum, and kept under barrier-filtered air conditions before and after all the experiments. The animal rooms were maintained on a 12-h
light-dark cycle at an air temperature of 23 ± 1.0°C and in a humidity of 55 ± 5.0%. The animal care included a weekly change of cages and a daily check of conditions during the animals’ lifetimes. All the experimental treatment were performed with the approval of the institution’s animal use committee.

Experimental design
For the preparation of soluble plutonium as described previously, a mixture of 10 mM 239Pu nitrate and 10 mM trisodium citrate at a molecular ratio of 1:50 was titrated to pH 6.8–7.2 by an addition of 1 N NaOH, diluted with physiological saline, and passed through 0.2- and 0.025-µm-pore Millipore filters to obtain a monomeric 239Pu citrate solution with a radioactivity ranging from 100 to 10,000 Bq per animal or with saline as the carrier control. They were kept in a closed-hood rack during their lifetimes. The cumulative skeletal dose with saline as the carrier control. They were kept in a closed-hood rack during their lifetimes. All the experimental treatment were performed with the approval of the institution’s animal use committee.

Histopathology
All control and 239Pu-injected animals were autopsied after death or killed on the occasion of a moribund state to examine the gross lesions of the main organs and skeletal bones. All organs were fixed in 10% phosphate-buffered formalin, and skeletal bones and bone tumors were then decalcified with a mixture of 10% formic acid and 10% neutral formalin. All tissue specimens were cut into small pieces, processed with graded ethanol and xylene in an automatic tissue processor, and embedded in paraffin to prepare 5- to 6-µm-thick sections on glass slides, stained with hematoxylin and eosin (H&E) for histopathological examinations using a light microscope. Differential diagnosis of bone tumors was performed either according to morphologic criteria or by some histochemical stainings as described. The other tumors, including hematopoietic and lymphoid neoplasms, were routinely diagnosed by morphologic criteria and immunohistochemical stainings as described previously.

Statistics
Survival periods with the competing risks of neoplastic or non-neoplastic death after the injection of 239Pu citrate were analyzed by the Kaplan-Meier method by using the log-rank and Peto-Wilcoxon tests as described previously. The standard errors (SE) for each tumor frequency in experimental groups were calculated according to the following formula, as described.

\[ SE = \sqrt{\frac{p(1 - p)}{N}} \]

where \( p \) = the proportion of animals with tumors and \( N \) = the total number of animals examined.

The significant differences between the control and the 239Pu-injected groups of mice were compared by the unpaired Student’s \( t \) test.

RESULTS
Survival reduction and early appearance of bone tumors after 239Pu-injection
As shown in Table 1, the survival periods after injections of 500 Bq or more of 239Pu citrate were significantly reduced in each strain of mice examined, and those of the groups of C3H and C57 mice injected with the lowest dose of 100 Bq were slightly longer but not significantly different from the controls. Such survival reduction was due to either neoplastic death caused by fatal tumors or non-neoplastic death caused mainly by acute or subacute hematopoietic dysplasias. The incidences of bone tumors were significantly higher in all groups injected with 100 Bq or more of 239Pu citrate than in those of the controls showing the minimum value, zero in each strain (Table 1). Lymphoid tumors, mainly systemic lymphomas, found in variable proportions of the control animals from each strain were, however, significantly reduced after 239Pu-injection, but they were slightly increased again in the higher dose groups injected with 5,000 or 10,000 Bq, even though their frequencies were still lower than those of the controls. In contrast, fewer or no myeloid leukemias were observed in all groups from each strain, and the other solid tumors affecting the soft tissues were significantly reduced after injections of 100 Bq or more of 239Pu citrate, and their frequencies reached zero over 1,000 Bq. These solid tumors included pulmonary adenomas, hepatocellular or cholangiocellular carcinomas, ovarian adenocarcinomas or granulosa cell tumors, dermal fibrosarcomas or histiocytomas, and mammary adenocarcinomas. Only a small number of bone tumors was found during the late period from 600 to 800 days after the injection of the lowest dose of 100 Bq, although all data on the dose and appearance time are not shown. As the number of tumor-bearers for each tumor from all the dose groups was plotted against every 100-day-interval after 239Pu-injection as shown in Fig. 1, most bone tumors appeared early during the period from 200 to 600 days after the injection in each strain, while all the other solid tumors appeared late, from 600 days or more. The appearance time of lymphoid tumors was almost coincident with that of bone tumors during the period from 200 to 600 days, except for C3H mice, in which lymphoid tumors appeared much earlier within 200 days after the injection. It was noteworthy that both the bone and lymphoid tumors did not concomitantly occur in the same animals. These findings indicate that significant survival reduction is due to the early appearance of bone tumors and some lymphoid tumors induced at higher injected doses of more than 5,000 Bq, while lymphoid and the other solid tumors were competitively reduced by the early increase of bone tumors.
Table 1. Summary of survivals and tumor frequencies in three strains of mice after injections of $^{239}\text{Pu}$ citrate.

<table>
<thead>
<tr>
<th>Injected dose (Bq)</th>
<th>Total No. of animals</th>
<th>Survival period (day)$^a$</th>
<th>C3H</th>
<th>C57</th>
<th>BC3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of tumor-bearers (% ± SE)$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bone</td>
<td>Lymphoid</td>
<td>Myeloid</td>
</tr>
<tr>
<td>0</td>
<td>60</td>
<td>763 ± 136</td>
<td>0</td>
<td>10 (16.7 ± 4.8)</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>30</td>
<td>808 ± 81</td>
<td>4 (13.3 ± 6.2)$^{**}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>30</td>
<td>592 ± 105*</td>
<td>19 (63.3 ± 8.8)$^{**}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,000</td>
<td>30</td>
<td>454 ± 72*</td>
<td>14 (46.7 ± 9.1)$^{**}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5,000</td>
<td>32</td>
<td>314 ± 50**</td>
<td>15 (46.9 ± 8.8)$^{**}$</td>
<td>2 (6.2 ± 4.2)$^{*}$</td>
<td>0</td>
</tr>
<tr>
<td>10,000</td>
<td>30</td>
<td>309 ± 67**</td>
<td>8 (26.7 ± 8.1)$^{**}$</td>
<td>3 (10.0 ± 5.5)</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$Mean ± SD of the survival periods after injections of saline (controls) or $^{239}\text{Pu}$ citrate in each group. Asterisks indicate significant differences in comparison to the controls (*$p < 0.05$, **$p < 0.01$).

$^b$Percent incidence ± SE in parenthesis of tumor-bearing animals out of total number of animals in each group. Asterisks indicate significant differences in comparison to the controls (*$p < 0.01$, **$p < 0.001$).

Fig. 1. The appearance time of bone, lymphoid, and other solid tumors from C3H (A), C57 (B), and BC3 (C) strain mice after injections of $^{239}\text{Pu}$ citrate. The columns indicate the number of tumor-bearers for each tumor from all the dose groups found in every 100-day-interval after $^{239}\text{Pu}$-injection.
Dose responses for the incidences of bone tumors after $^{239}$Pu-injection

The dose response curves in Fig. 2 implicate that the significantly higher bone tumor induction was noted in all the strains of mice at the lowest skeletal doses of 0.6 to 0.7 Gy, in comparison to the control value, zero. Moreover, their incidences increased sharply from 1 Gy, reached a maximum of 50 to 63% at doses of 2 to 3 Gy, then dropped to the lower incidences of 16 to 31% at doses over 10 Gy. Such a bell-shaped pattern of dose responsiveness was almost similar among three strains of mice, though small differences were noted in the dose ranges and magnitudes for the maximum responses. The reduced incidences of bone tumors at higher skeletal doses resulted mainly from non-neoplastic death by acute or subacute hematopoietic dysplasias or partly from lymphoid neoplasms at the higher doses (data not shown), since none of the other solid tumors were found in animals that died as early as 200 days at higher dose ranges of more than 5,000 Bq or 10 Gy (Table 1 and Fig. 1).

Macroscopic and microscopic appearances of bone tumors after $^{239}$Pu-injection

The primary bone tumors found macroscopically (larger than 5 mm in diameter) or microscopically (smaller than 2 mm in diameter) were distributed in almost the same skeletal bone sites of the three strains of mice (Fig. 3). Thus, bone tumors were distributed in the thoracic and lumbar vertebra with the highest proportions (46 to 54%) to all the bone tumors, the second higher proportions (20 to 24%) in the femur and tibia, and the smaller proportions (10 to 16%) in the ischium and pelvis, or humerus and scapula. No tumors were found in the other skeletal bone sites, such as the cranium and radius, except for submandibular bones from one C57 strain mouse. No clear differences in the skeletal distribution sites were seen among experimental groups with different injected or skeletal doses (data not shown).

Although the macroscopic sizes of bone tumors varied from 5 mm to 30 mm in diameter, their occurrence was mostly single and unilateral in the skeletal bones, and gross appearances were mostly expressed as being white or yellowish-colored, variably calcified, and smooth- or rough-surfaced tumor masses. Histopathological features of these bone tumors include fewer osteomas characterized by the relatively regular growth of spindle-shaped osteoblasts along with trabecular bone formation (Fig. 4A) and mostly osteogenic sarcomas with a variety of growth patterns and predominant cellular or stromal components. Many osteosarcomas were characterized by an irregular growth of large polygonal osteoblasts with osteoid and trabecular bone formations (Fig. 4B), whereas some osteosarcomas showed fibroblastic or histiocytic appearances accompanied with fibrous connective tissue stromas (Fig. 4C), or predominant cellular growth along endosteal bone surfaces and invasion into the bone marrows (Fig. 4D). A few cases of osteogenic sarcomas were rich in multinucleated giant cells or appeared to be giant cell osteogenic sarcomas with a predominant growth of osteo-
clast-like giant cells (Fig. 4, E and F). Although they had no obvious relations to the injected doses or skeletal doses, metastatic osteogenic sarcomas were occasionally found in the lung, liver, kidney, spleen, or regional lymph nodes. Morphological features of these metastatic osteosarcomas were sometimes different from those of the primary site (Fig. 4, G and H).
DISCUSSION

The present results implicated that survival reduction as the administered doses increased was commonly observed in three mouse strains that have been found to show different carcinogenic responses before and after external exposures to ionizing radiations, and also that the cause of death was mostly the bone tumors increased significantly during the earlier periods, though a few were due to the early onsets of lymphoid neoplasms or non-neoplastic, hematopoietic dysplasias at higher dose ranges. Even though different isotopes, chemical forms, and entry routes were applied to experimental animals, alpha-emitting bone-seekers predominantly induced bone tumors, which were the most common causes of death, occurring early after either the inhalation exposures of beagle dogs to $^{238}$PuO$_2$ aerosols$^{15,16}$, injections of $^{224}$Ra into the dog$^{17}$, or injections of $^{237}$Np into rats$^{18}$. The other solid tumors affecting the liver, ovary, lung, and skin or mammary glands, rather decreased much less in $^{239}$Pu-injected mice than in the controls, and they appeared as late as the spontaneous occurrence in the control animals. This should be a result of the competition with the early occurrence of bone and a few lymphoid tumors, as described previously$^{19}$. It also implies that most of the other solid tumors, if observed, were spontaneous but not significant causes of death, suggesting the differences in the dose distribution and target organs from low LET radiation exposures$^{19}$. Lymphoid neoplasms, which are frequently observed in mice before or after low LET radiation exposures, were not, however, significantly induced; they slightly increased during the early periods after the injections of higher doses in which bone tumors were rather reduced. Although these findings are not consistent with those of mice after fractionated $^{223}$Ra-injections$^{20}$, which induced early malignant lymphomas with an incidence of 13.5%, followed by the late occurrence of osteosarcomas with an incidence of 7%, the protracted exposures to low-dose alpha particles with lower dose rates should be important for the induction of lymphoid neoplasms. Whereas spontaneous murine bone tumors are only speculated to be closely associated with lymphomas, but not with the other solid tumors because of a common viral agent or competing risks in CFI strain$^{21}$, it might be conceivable that lymphoid neoplasms after alpha radiations appear to be indirectly induced by some damaged but still rescued hematopoietic stem cells that migrated into peripheral lymphoid tissues. Myeloid leukemias, in contrast, were much less or scarcely observed in the control and $^{239}$Pu-injected mice from three strains, even though lower but significant incidences of myeloid leukemias could be yielded in CBA/H mice after fractionated injections of lower doses of $^{239}$Pu or $^{226}$Ra$^{22,23}$. Taken together, only the bone tumor is the major malignancy specific to $^{239}$Pu-injection; the other solid tumors and lymphomyeloid neoplasms do not result directly from the internal exposures of the committed organs to alpha particles.

The bell-shaped dose response curves, characterized by significant increases at skeletal doses of more than 0.6 to 0.7 Gy, peak incidences at 2 to 3 Gy, and declines over 10 Gy, were commonly noted in three different mouse strains, though slight differences were present in the dose ranges and magnitudes for maximum responses. Moreover, the distribution pattern of macroscopic and microscopic bone tumors was almost similar in three strains of mice because approximately 70 to 80% of primary tumors were found in the vertebra, femur, and tibia, and the remaining 10 to 20% were distributed in the ischium, pelvis, humerus, and scapula. Interestingly, all of these skeletal bone sites have well-developed trabecular bone surfaces with a higher rate of trabecular bone formation, and large vascular sinusoids with discontinuous endothelial linings as described in the beagle dogs$^{24,25}$. Thus, based on the findings that metabolic behavior and toxicity ratio of $^{239}$Pu in the mouse skeletal bones are almost similar to those of the dog$^{5,7}$, it is noteworthy that such anatomical structures ensure initial contact with the endothelium along closed capillary beds, and then a higher deposition of plutonium on trabecular bone surfaces, resulted in a more effective irradiation of target osteoblasts for carcinogenic processes. Histological appearances of bone tumors, mostly osteosarcomas, were characterized commonly in all the strains of mice by an irregular growth of osteoblasts along or inside endosteal bone surfaces accompanied by trabecular bone formation. All of these findings, taken together, indicate again that osteosarcoma is only the neoplasm specifically and commonly induced by alpha particles emitted from plutonium deposited on the trabecular bone surfaces independent of animal species or strains. Concerning the strain differences, the genetic background of mouse strains may influence the latent periods and metastasis of osteosarcomas induced by $^{223}$Ra, as described$^{26}$. The radiosensitivity for bone cancer induction in dogs following an injection of $^{233}$Pu or $^{226}$Ra is closely related to the skeletal dose distribution, which depends on breed-specific bone mass and deposition of radioactivity$^{27}$, or on age-related bone metabolism$^{28}$, but not on body size$^{29}$. In this respect, the susceptibility of three strains of age-matched mice to bone tumorigenesis, as expressed by the order of $\text{C3H} < \text{BC3} < \text{C57}$, might be due to strain-specific differences in bone mass and the skeletal distribution of $^{239}$Pu. In comparison to $^{239}$Pu and other alpha-emitting bone seekers, the other radiation sources, even beta-emitting bone seekers induce less effectively bone tumors. Only a single injection of 50 to 100 times higher doses of $^{89}$Sr than those of alpha emitters resulted in osteosarcomas in mice with the highest incidence of approximately 60 to 70%, as well as with increased frequencies of hemopoietic and vascular neoplasms$^{30}$. The inhalation exposures of dogs to soluble $^{144}$Ce chloride induced liver, lung, and nasal mucosal neoplasms rather than primary bone tumors, despite the highly skeletal deposition$^{31}$. It seems more difficult to induce osteosarcomas in mice by chemical carcinogens as described elsewhere$^{32}$, though spontaneous osteosarcomas with very low incidences were observed in a colony of C57BL mice, and transplants from some primary tumors were bone forming, suggesting the relations of viral agents$^{33}$. Evi-
idence for the viral etiology of murine osteosarcomas was demonstrated first in CF1 mice by Finkel et al. who have developed the experimental model for carcinogenesis to introduce pathogenetic mechanisms common to Sr- and virus-induced osteosarcomas. The murine osteosarcoma virus termed FBJ or FBR-MSV has been characterized to bear a transforming sequence of fos protooncogenes, and the recent study further revealed that deregulated c-fos or v-fos expression in bone cells induces tumorigenicity and the other factors related to metastasis. The molecular basis for Pu-induced bone tumorigenesis, however, remains to be fully elucidated, but would be responsible for the largest and specific carcinogenicity of plutonium compounds among bone seekers. In conclusion, the injection of soluble Pu citrate most effectively and specifically induces osteogenic sarcomas commonly in three strains of mice, irrespective of genetic background for carcinogenesis, with almost similar patterns of dose responsive, skeletal distribution sites, and histopathological features. Except for lymphoid neoplasms that appeared as early as bone tumors at the higher dose ranges after Pu-injection, the other solid tumors and myeloid leukemias were rather reduced, or reduced much less than the controls, because of the competition with bone tumors, suggesting that neoplasms besides the bone tumors showed non-specific and spontaneous occurrence after the injection of soluble plutonium compounds.

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