Immunohistochemical Study on Cellular Origins of Rat Lung Tumors Induced by Inhalation Exposures to Plutonium Dioxide Aerosols as Compared to Those by X-ray Irradiation

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Immunohistochemistry / Rat / Lung tumors / Plutonium-exposure / X-irradiation

Immunohistochemical examinations were performed on rat pulmonary tumors induced by inhalation exposures to $^{239}$PuO$_2$ aerosols, or by X-ray-irradiation to identify and compare cellular origins or, in turn, target cells at risk for radiation carcinogenesis. Both plutonium-induced and X-ray-induced pulmonary tumors appeared to occur from the lower respiratory tract epithelium through bronchioles into alveoli, and were histopathologically diagnosed as adenoma, adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma. Immunohistochemical staining of neoplastic lesions using rabbit polyclonal antibodies to rat surfactant apoprotein A specific for alveolar type II pneumocytes, and Clara cell antigen specific for nonciliated bronchiolar Clara cells, showed that most of the adenomatous and adenocarcinomatous lesions from plutonium-exposed or X-irradiated rats were positive for either or both antigens, while, in contrast, adenosquamous and squamous lesions were mostly negative for both antigens. Even though there were some differences in the proportions and distributions of immunoreactive cells between plutonium- and X-ray-induced tumors and among neoplastic lesions, the results indicate that radiation-induced pulmonary adenomas and adenocarcinomas mostly originate from either alveolar type II pneumocytes or bronchiolar Clara cells, while adenosquamous and squamous carcinomas may be derived from the other epithelial cell components, or might have lost specific antigenicity during their transforming differentiation.

INTRODUCTION

In risk assessments of radiation carcinogenesis, it is essentially important to identify the cellular origins of radiation-induced tumors for determining target cells at risk, and for more accurate dose estimation to induce neoplastic changes in the most sensitive cells.

In our previous studies on pulmonary carcinogenesis in rats following inhalation exposures to an alpha-emitting radionuclide, $^{239}$PuO$_2$ aerosols, most predominantly induced primary lung tumors were epithelial types derived from the lower respiratory tract epithelium through bronchioles into alveoli, such as adenomas and adenocarcinomas, or, to a lesser degree, adenosquamous and squamous cell carcinomas$^{1)}$. Initial pulmonary damage of the bronchialalveolar lining epithelium resulted in hyperplastic and metaplastic lesions that finally transformed to adenomas and adenocarcinomas in 6–12 months after inhalation exposures$^{2)}$. Such findings implicate that the majority

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of lung tumors from the lower respiratory tract epithelium originate from nonciliated bronchiolar Clara cells and/or alveolar type II pneumocytes as described to be radiosensitive precursor cells at risk for lung tumors.\(^3,4\) It is, however, still uncertain or controversial what types of cells are the origins of experimentally induced pulmonary epithelial tumors, and whether there are any differences in the cellular origins of tumors among radiation sources and chemical carcinogens for the initiation and/or promotion of tumors. Concerning the cellular origins of plutonium-induced rat lung tumors, Clara cells appear to be possible target cells for histogenesis of bronchogenic or bronchioloalveolar carcinomas by light and electron microscopic studies,\(^5,6\) whereas immunohistochemistry combined with electron microscopy of both preneoplastic and neoplastic lesions implied more clearly the major origin of lung tumors from type II pneumocytes.\(^7\) Although such differences in demonstration and estimation of cellular origins are resulted from the methodological approach, they could also be based on the pulmonary deposition of inhaled aerosols, and the dose distribution throughout the respiratory system, which is dependent on the aerosol particle size and physicochemical states of inhaled plutonium.\(^1,3,4\) Thus, submicron-sized aerosols tend to deposit in the lower respiratory tract regions, i.e. bronchial-bronchiolar to alveolar epithelium, which should be most likely to be targeted by alpha particles, while a more soluble form of inhaled aerosols could be distributed more uniformly in the whole lung lobes, so that target cells should be more widely distributed from bronchial to alveolar epithelium. In addition, the susceptibility of target cells for radiation carcinogenesis could affect phenotypic clonalities and differentiation of tumor cells, although this has not yet been clarified.

In the present study, we performed immunohistochemical examinations on primary lung tumors from \(^{239}\)PuO\(_2\)-exposed rats to detect either a type II cell- or a Clara cell-specific antigen, and compared the pattern of positive staining and the distribution of antigens with those of X-ray-induced primary lung tumors. The results are discussed on the cellular origins of radiation-induced lung tumors, as compared to the previous data including human and experimentally induced lung tumors.

### MATERIALS AND METHODS

#### Animals

Female Wistar (W/M) strain rats, purchased from a breeding colony of the National Institute of Radiological Sciences, were used for either inhalation exposures to plutonium dioxide aerosols or X-ray-irradiations at the age of 100 to 120 days after birth. Before and after the experiments, animals were housed 5 per polycarbonate cage and kept under a barrier-filtered air condition with a commercial diet (Funabashi Farm Co.) and water \textit{ad libitum}. The animal rooms were maintained on a 12-hr light:dark cycle at 23±1.0°C with a humidity of 55±5.0%. The animal care included a weekly change of cages and a daily check of the animals’ conditions during their lifetimes. All the experimental treatments were performed with the approval of the institution’s animal use committee.

#### Experimental Treatments

For inhalation exposures to plutonium(Pu) aerosols, nose-only inhalation exposures to submicron-sized and polydispersed aerosols (AMAD of 0.40±0.005 µm with GSD of 2.2±0.1 µm) of high-fired \(^{239}\)PuO\(_2\) were performed to achieve an initial lung deposition of 30 to 3000 Bq and a mean absorbed lung dose of 0.5 to 12 Gy, as previously described\(^1,2\). The mean survival time (day) ± SD and the mean lung dose (Gy) ± SD of Pu-exposed rats selected for immunohistochemical examinations on lung tumor specimens were 728±159 and 3.38±2.23 (n=135), respectively. For X-ray-irradiations, either whole-body, fractionated irradiations with a dose rate of 0.1 Gy/min or local (thoracic), single irradiations with a dose rate of 0.6 Gy/min were performed under the condition of 200 keV and 10–20 mA, with a filter of 0.5 Cu and 0.5 Al and with FSD of 75–100 cm using Pantak HF 320S (Shimadzu) to achieve an accumulated dose of 0.5 to 10 Gy (unpublished). The mean survival time (day) ±
SD and the mean lung dose(Gy) ± SD of both whole-body and local X-irradiated rats selected for immunohistochemical examinations on lung tumor specimens were 718±131 and 5.60±3.07 (n=41), respectively.

**Histopathological Examinations**

All of the dead or moribund and sacrificed animals were autopsied, and the main organs, including the lungs, were fixed in 10% phosphate-buffered formalin, dehydrated, and embedded in paraffin. Then, 5-µm-thick serial sections were prepared from each lobe, and one of them was stained with hematoxylin and eosin (HE) to be examined under a light microscope for histopathological diagnoses of primary lung tumors, as previously described. Almost all histopathological types of lung tumors from both Pu-exposed and X-irradiated rats were epithelial types, being classified into adenoma (AD), adenocarcinoma (AC), adenosquamous carcinoma (ASC) and squamous cell carcinoma (SCC). In the present study, only lung tumor specimens with little postmortem changes were selected from Pu-exposed and X-irradiated rats for immunohistochemical staining; each of the numbers and diagnoses of histopathological types are as given in Table 1.

**Immunohistochemistry**

Immunohistochemical staining of lung tumors selected from both Pu-exposed and X-irradiated rats was performed using an avidin-biotin-immunoperoxidase complex (ABC) method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with rabbit polyclonal antibodies to either surfactant apoprotein A(SP-A) specific for rat type II pneumocytes and rat Clara cell 10-kd protein (CC-10) specific for nonciliated terminal bronchiolar Clara cells, both of which were courteously given by Dr. Gurmukh Singh, University of Pittsburgh, PA. Briefly, unstained 5-µm-thick paraffin sections were deparaffinized with xylene and graded ethanol, rehydrated, and pretreated with 0.3% hydrogen peroxide in methanol at room temperature for 30 min to inactivate the endogenous peroxidase activity. After washing with 0.05% Tris (pH 7.6)-buffered saline (TBS), sections were incubated with normal goat serum at room temperature for 1 hr to block any nonspecific Fc-binding of a secondary antibody, and then incubated with each of the primary antibodies (SP-A diluted 1:200 and CC-10 diluted 1:400) at 4°C for overnight. After washing with TBS, sections were reacted with biotinylated secondary goat anti-rabbit IgG antibody at room temperature for 1 hr, and then coupled with ABC at room temperature for 30 min. After washing with TBS, sections were treated with the substrate, 3,3′-diaminobenzidine tetrachloride (DAB), to visualize the immunoreactivity, and counterstained with hematoxylin. As negative controls, other sections were only incubated with normal rabbit serum instead of primary antibodies under conditions similar to those described above. As positive controls, normal alveolar epithelium and the terminal bronchiole surrounding neoplastic lesions of the stained sections were examined respectively for the staining reactivity of type II pneumocytes and Clara cells. Actually, almost all normal alveolar type II and bron-

### Table 1. List of the numbers and histopathological types of lung tumors examined.

<table>
<thead>
<tr>
<th>Lung tumor types</th>
<th>Pu-exposed</th>
<th>X-irradiated*</th>
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<tbody>
<tr>
<td>Adenoma (AD)</td>
<td>14</td>
<td>17 ( 3+14)</td>
</tr>
<tr>
<td>Adenocarcinoma (AC)</td>
<td>65</td>
<td>18 ( 9+ 9)</td>
</tr>
<tr>
<td>Adenosquamous carcinoma (ASC)</td>
<td>37</td>
<td>1 ( 1+ 0)</td>
</tr>
<tr>
<td>Squamous cell carcinoma (SCC)</td>
<td>19</td>
<td>5 ( 0+ 5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>135</td>
<td>41 (13+28)</td>
</tr>
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</table>

* No. of cases from whole body irradiations(left) and local (thoracic) irradiations(right), respectively in parenthesis
chiolar Clara cells, respectively, showed strongly diffuse or granular staining patterns within their cytoplasms, while most neoplastic lesions were variably stained, with slight to moderate intensity, with weakly and partly positive patterns, and at almost negligible levels. Thus, to evaluate immunostaining with SP-A and CC-10, each neoplastic lesion was observed with a light microscope to detect and assess the immunoreactivity based on the following criteria: (–) as negative staining in 90% or more, and (+) as positive staining in 20% or more of at least 2,000 neoplastic cells randomly counted in 20 high-power (×400) fields. All the neoplastic lesions were finally classified into SP-A or CC-10 single positive (SP-A+/CC-10⁻ or SP-A⁻/CC-10⁺), double positive (SP-A+/CC-10⁺) or double negative (SP-A⁻/CC-10⁻).

**RESULTS**

**Morphological and Immunohistochemical Appearances of Neoplastic Lesions**

As described above, all the neoplastic lesions examined on the selected lung tumor specimens from both Pu-exposed and X-irradiated rats were classified into four histological types: adenoma (AD), adenocarcinoma (AC), adenosquamous carcinoma (ASC) and squamous cell carcinoma (SCC). The morphological appearance of each neoplastic lesion was similar in both Pu-exposed and X-irradiated rats; however, the number and size of lesions distributed in the lung were smaller in X-irradiated rats than in Pu-exposed rats, and were larger under local (thoracic) X-irradiations than under whole-body X-irradiations (data not shown). Ductal and papillary forms of AD lesions were most predominantly located in peripheral, and subpleural, or peribronchiolar regions. AC lesions were mostly ductal and papillary, but partly solid and compact, and were distributed more extensively from peripheral and subpleural regions into deep bronchial-bronchiolar and alveolar regions than AD lesions. ASC lesions, showing solid and compact features, were more frequently distributed in peribronchial or peribronchiolar regions adjacent to AC lesions, where-
rats (Table 2). In the lung tumors from Pu-exposed rats, 56% (59 out of 105 neoplastic lesions examined) of the AD lesions showed double positive staining with both SP-A and CC-10, while either 19% (20 out of 105) or 23% (24 out of 105) of AD lesions was SP-A or CC-10 single positive, respectively. A similar trend, but slightly different distribution of immunostaining patterns, was noted for AC lesions of lung tumors from Pu-exposed rats; almost half, 46% (54 out of 118), of the AC lesions were SP-A single positive and 36% (43 out of 118) were SP-A and CC-10 double positive, while only 10% (12 out of 118) were

Fig. 1. Light microscopy of $^{239}$PuO$_2$-induced rat lung tumors. Papillary adenoma stained with HE (A), and its positive immunostaining with SP-A antibody (B). Ductal adenocarcinoma stained with HE (C) and its positive immunostaining with CC-10 antibody (D). Papillary adenoma stained with HE (E), and its positive immunostaining with SP-A antibody (F) and CC-10 antibody (G).
CC-10 single positive. On the other hand, most of the ASC (77%) and SCC (96%) lesions were SP-A and CC-10 double negative, except that 15% (9 out of 58) of the ASC lesions were SP-A single positive, and only 5% (3 out of 58) of ASC lesions were CC-10 single positive. Only 3.8% (2 out of 52) of the SCC lesions were SP-A single positive. In the lung tumors from X-irradiated rats, almost a third to a half, 37% (19 out of 51) and 43% (22 out of 51) of the AD lesions were CC-10 single positive or SP-A and CC-10 double positive, respectively, while 63% (22 out of 35) of AC lesions were SP-A and CC-10 double positive (Table 2). The remaining 10 to 14% each of the AD and AC lesions showed either single positive or double negative staining with SP-A and CC-10. All of the ASC and SCC lesions, in contrast, showed double negative staining with SP-A and CC-10, although the total numbers of ASC and SCC lesions examined were fewer than those of the AD and AC lesions. Such differences in the distribution of the immunostaining patterns among histological types of neoplastic lesions and between lung tumors from Pu-exposed and X-irradiated rats were more clearly shown by expressing as either SP-A or CC-10 positive lesions (Fig. 3). Thus, in lung tumors from Pu-exposed rats, almost 75% to 80% of the AD lesions were either SP-A or CC-10 positive, and 82% of the AC lesions were SP-A positive, while 47% of the AC lesions were CC-10 positive (Fig. 3A). In lung tumors from X-irradiated rats, 53% to 80% of the AD lesions showed SP-A or CC-10 positive staining, respectively, while almost two-thirds, 74% of the AC lesions were positive with SP-A or CC-10 (Fig. 3B). Taking into account for the differences in both findings, the AD lesions from X-irradiated rats appeared to be CC-10 positive, while the AC lesions from Pu-exposed rats preferentially showed SP-A pos-

Fig. 2. Light microscopy of X-ray-induced rat lung tumors. Papillary adenocarcinoma stained with HE (A), and its positive immunostaining with SP-A antibody (B). Ductal and papillary adenoma stained with HE (C) and its positive immunostaining with CC-10 antibody (D).
itive staining. Contrary to this, the AD lesions from Pu-exposed rats and the AC lesions from X-irradiated rats almost equally showed highly positive staining with both SP-A and CC-10. Except for SP-A positive staining (17%) of ASC lesions in lung tumors from Pu-exposed rats, most or all of the ASC and SCC lesions from both Pu-exposed and X-irradiated rats showed negative staining with both SP-A and CC-10.

### Table 2. Immunostaining of pulmonary neoplastic lesions from $^{239}$PuO$_2$-exposed and X-irradiated rats.

<table>
<thead>
<tr>
<th>Neoplastic lesions examined</th>
<th>SP-A/CC-10 immunostaining (%)$^a$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>Adenoma</td>
<td>105</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>118</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>58</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>52</td>
</tr>
</tbody>
</table>

$^a$ No. of lesions with SP-A single positive (+/–), CC-10 single positive (–/+), SP-A and CC-10 double positive (+/+ or double negative (–/) staining, and percentage in total numbers of each neoplastic lesion examined in parenthesis.

Fig. 3. Distribution of SP-A positive and CC-10 positive neoplastic lesions in rat lung tumors induced by inhalation exposures to $^{239}$PuO$_2$ (A) and by X-ray-irradiation (B). The columns indicate the percentage of neoplastic lesions positively stained with either SP-A or CC-10 antibody in the number of each neoplastic lesion showing adenoma (AD), adenocarcinoma (AC), adenosquamous carcinoma (ASC), and squamous cell carcinoma (SCC).
(Fig. 3A&B). These results indicate that the AD and AC lesions were mostly SP-A and/or CC-10 positive in radiation-induced rat lung tumors, although some differences in the distribution of immunostaining patterns were present between lung tumors from Pu-exposed and X-irradiated rats. ASC and SCC lesions, however, showed nearly negative staining with SP-A or CC-10.

**DISCUSSION**

Our previous studies\(^1\,^2\) have implicated that most pulmonary tumors induced in rats by inhalation exposures to submicron-sized and polydispersed \(^{239}\)PuO\(_2\) aerosols were adenomas and adenocarcinomas, both of which were distributed in the lower pulmonary regions, and therefore thought to arise from the bronchioloalveolar lining epithelium, such as nonciliated bronchiolar Clara cells and/or alveolar type II pneumocytes. The present immunohistochemical analysis revealed that almost 80% of adenomas showed either type II cell or Clara cell origins, while the adenocarcinomas appeared to be derived from type II pneumocytes (Fig. 3A). These results differ from those of another study on plutonium-induced rat pulmonary tumors, which indicated a majority from type II pneumocytes with the exception of one adenosquamous carcinoma being positive and all the squamous cell carcinomas being negative for both a surfactant apoprotein and a Clara cell antigen\(^7\). Double positive staining with both type II and Clara cell antigens was also observed in our cases of adenomas and adenocarcinomas (56% and 36% respectively), suggesting that some parts of pulmonary tumors could concomitantly express both antigens in a certain differentiation state despite their origins either from type II or Clara cells. We supposed that most neoplastic cells became anaplastic toward immature embryonic stages; this, however, is not surprising because of evidence that embryonic murine type II pneumocytes and Clara cells are derived from the same stem cells\(^10\). Mason et al. \(^11\) showed that only SP-C among a series of isotypic surfactant apoproteins was specific for type II cells in normal murine lungs, while the remaining SP-A, SP-B and SP-D apoproteins were expressed in both type II cells and CC-10 positive Clara cells. From these results, it may be indicated in the present study that SP-A single positive cells should be type II cells, while double positive cells and CC-10 single positive cells could be Clara cells under a differentiated, but also neoplastic state. Most of the adenosquamous and squamous cell carcinomas from Pu-exposed rats were negative for both antigens, except for only a small part (15%) of the adenosquamous carcinomas, which could be derived from type II cells. This suggests that the origins of pulmonary tumors characterized with squamous metaplasias should be cells other than type II and Clara cells, or that type II or Clara cell-specific antigens expressed during the onset of tumor cells may have disappeared under differentiation processes into other cell types\(^7\). The latter possibility is likely to occur during a transition in certain genetic and immunohistochemical components of human non-small cell pulmonary carcinomas among the adenocarcinomas, adenosquamous carcinomas and squamous cell carcinomas\(^12\).

The present study also implied the cellular origins of X-ray-induced rat pulmonary tumors, although the total numbers examined were much fewer than those of Pu-induced tumors. As compared to Pu-induced pulmonary tumors, the only differences were in the proportions of SP-A and CC-10 positive lesions between adenomas and adenocarcinomas, as shown in Fig.3B. Almost 80% of the adenocarcinomas could originate from either type II or Clara cells, while the adenomas would be derived from Clara cells rather than type II cells. Such differences from those of Pu-induced tumors are not, however, significant because less than 14% of the adenomas or adenocarcinomas, and all of the adenosquamous and squamous cell carcinomas were double negative for both antigens. This suggests that most of the adenomas and adenocarcinomas from X-irradiated rats originate from either type II or Clara cells, and that adenosquamous and squamous cell carcinomas derive from other cell types, or have lost type II or Clara cell-specific antigenicity, as described above in Pu-induced tumors. Taken together
with those findings on the cellular origins of radiation-induced pulmonary tumors including $^{239}$Pu$^{6,7}$ and other $\alpha$-emitter, $^{210}$Po$^{13}$, the cellular origins of bronchioloalveolar adenomas and adenocarcinomas from radiation-exposed animals are mostly type II pneumocytes and/or Clara cells. In this respect, both ICRP$^3$ and NCRP$^4$ have described these cells as being radiosensitive precursor cells at risk for radiation-induced lung tumors, but have not referred to the other cell types for the candidate origins of squamous cell carcinomas. In this regard, the upper respiratory tract epithelium could also be susceptible for radiation carcinogenesis based on the experimental evidence that rat tracheal epithelial cells showed a preneoplastic transformation in vitro by $\alpha$- or $\gamma$-irradiations$^{14}$.

Numerous investigations of the cellular origins of chemically induced pulmonary tumors in small rodents have, however, resulted in controversy. Type II pneumocytes-derived lung tumors have been noted in N-nitrosomethylurea (NMU)-induced adenomas and carcinomas from F344 rats$^{15}$, N-nitrosodiethylamine (DEN)-induced adenomas and adenocarcinomas from B6C3F1 and A mice$^{16}$, or transplacentally N-nitrosourea (ENU)-induced adenomas and carcinomas from C3H and Swiss mice$^{17}$, while a part of transplacentally N-nitrosourea (ENU)-induced adenomas and carcinomas from Swiss mice$^{18}$, N-nitrosodiethylamine (NDEA)-induced bronchiolar carcinomas from Syrian golden hamsters$^{19}$, and NMU- or ENU-induced bronchiolar squamous cell or adenosquamous carcinomas from Swiss mice$^{20}$ have been reported to originate from Clara cells. Such diversity concerning the cellular origins of chemically induced lung tumor models in animals may reflect the sensitivity of target epithelial cells, and their morphologic and biochemical plasticity after arising from multipotent stem cells, as have been referred to murine urethane-induced solid (type II cell) and papillary (Clara cell) adenomas$^{21,22}$. It should be noted that there have also been descriptions on the type II cell-origins of human bronchioloalveolar carcinomas$^{23-25}$, while only a part of the bronchioloalveolar and squamous cell carcinomas appears to have phenotypic expression specific for Clara cells$^{26,27}$. Since even human Clara cells as well as murine ones$^{11}$ could produce surfactant apoproteins, it may be plausible that some bronchioloalveolar carcinomas, even if they were originated from Clara cells, had lost the ability to produce Clara cell antigens, but, instead, increased the ability to produce surfactant apoproteins during their transformation$^{28}$. Such phenotypic changes from Clara cells into type II cells, or the reverse could result in a variety of cellular origins of pulmonary tumors from experimental animals as well as humans.

In conclusion, except for the small differences in the proportions and distributions of type II or Clara cell antigens among neoplastic lesions from Pu-exposed and X-ray-irradiated rats, most of the radiation-induced pulmonary adenomas and adenocarcinomas originate from either alveolar type II pneumocytes or bronchiolar Clara cells, while the majority of adenosquamous and squamous cell carcinomas may be derived from the other epithelial cell components, or might have lost antigenicity specific for either type II or Clara cells during their transforming differentiation.

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