Serial Sacrifice Study of Pathogenesis of $^{210}$Po-Induced Lung Tumors in Syrian Golden Hamsters

Ann R. Kennedy, Robert B. McGandy, and John B. Little

Harvard University, School of Public Health, Department of Physiology, Boston, Massachusetts 02115

ABSTRACT

The pathogenesis of $^{210}$Po-induced tumors in the peripheral lung of Syrian golden hamsters has been studied in a serial sacrifice experiment utilizing both plastic (glycol methacrylate) and routine paraffin embedding procedures for lung sections. A rapid progression from hyperplasia was performed during and after a course of intratracheal instillation of $^{210}$Po.

INTRODUCTION

$^{210}$Po is an $\alpha$-radiation-emitting radionuclide that is present in cigarette smoke (29) and the lungs of cigarette smokers (25) and has been implicated as a causative factor in human lung cancer from cigarette smoke (23). We have previously shown intratracheally administered $^{210}$Po to be a potent lung carcinogen in Syrian golden hamsters, with tumor induction time and incidence related to dose over a broad range (24). $^{210}$Po is carcinogenic to hamsters at whole-lung doses as low as 15 rads (23).

The tumors arise in the bronchiolo-alveolar region of the lung and have been classified as combined epidermoid and adenocarcinomas (24). By means of thin plastic (glycol methacrylate) sections, electron microscopy, and histology, we have previously concluded that the bronchiolar Clara cell is the probable cell of origin of the $^{210}$Po-induced tumors (13, 15, 16). The $^{210}$Po-induced peripheral lung tumors are similar in appearance to those induced by other radionuclides (1, 3), chemical carcinogens (33), and oncogenic viruses (18). In humans, bronchioloalveolar carcinoma is a peripheral lung tumor similar in appearance to the $^{210}$Po-induced tumor in hamsters (6, 15, 19); it is estimated to represent as much as 8% of all human lung tumors (2), and there is some evidence that smoking plays a significant role in its pathogenesis (4).

In our previous $^{210}$Po tumor induction studies, it was difficult to determine the pathogenesis of the tumors since the lungs of animals that died during such experiments showed only end-stage disease. This experiment was designed to study the sequential stages in the development and extent of benign and malignant changes in the lungs of hamsters exposed to $^{210}$Po. A serial sacrifice experiment was performed during and after a course of intratracheal instillations of either $^{210}$Po in 0.9% NaCl solution or $^{210}$Po adsorbed onto ferric oxide carrier particles. At each sacrifice lungs from some of the animals were embedded routinely in paraffin, and lungs from other animals were embedded in plastic (glycol methacrylate) to allow high resolution in the identification of cell types. The lungs of animals that died during the experiment were embedded in paraffin for a comparison with the lungs of animals sacrificed at similar times. A preliminary report of some of these data has been presented previously (21).

MATERIALS AND METHODS

Random-bred, male Syrian golden hamsters (Dennen Animal Industries, Gloucester, Mass.) weighing 100 to 125 g were given 7 intratracheal instillations of approximately 0.05 $\mu$Ci $^{210}$Po or 0.1 $\mu$Ci $^{210}$Po bound to 3-mg ferric oxide carrier particles in 0.2 ml 0.9% solution (hereafter called $^{210}$Po-ferric oxide group) as described by Little and O'Toole (24). On the basis of radiochemical analyses of the lungs of animals sacrificed during the course of the experiment, the approximate dose to the whole lung for the group given $^{210}$Po in 0.9% NaCl solution (hereafter called $^{210}$Po-0.9% NaCl group) was 1000 rads, and for the $^{210}$Po-ferric oxide group the dose was 2700 rads. From each treatment group 1 or 2 animals were sacrificed 7 days after each $^{210}$Po instillation and then following the instillation period at frequent intervals and were used for the preparation of 1-µm plastic (glycol methacrylate) sections. Animals from each treatment group were also sacrificed at similar times for routine paraffin section analysis of their lungs.

Paraffin sections were made from the lungs of all animals that died during the course of the serial sacrifice experiment. A total of 375 hamster lungs were analyzed in these experiments: 63 from the $^{210}$Po-0.9% NaCl plastic section serial-sacrifice group, 59 from the $^{210}$Po-ferric oxide plastic section serial-sacrifice group, 52 from the $^{210}$Po-ferric oxide paraffin section serial-sacrifice group, and 56 from the $^{210}$Po-ferric oxide paraffin section serial-sacrifice group. Paraffin lung sections were analyzed from the 145 animals that died during the experiment: 60 from the $^{210}$Po-0.9% NaCl group and 85 from the $^{210}$Po-ferric oxide group.

Animals were sacrificed by Brevaltal sodium (Eli Lilly and Co., Indianapolis, Ind.) overdose and exsanguination from a renal artery. For the preparation of plastic sections of lungs, the diaphragm was punctured and the trachea and collapsed lungs were removed en bloc, wrapped in 0.9% NaCl solution-soaked gauze, and evacuated at -740 mm Hg for 10 min. The trachea was cannulated with PE190 polyethylene tubing, and the lungs were filled at a pressure of 30 cm of water with glutaraldehyde fixative (3% glutaraldehyde in TC-199, x1; Grand Island Biological Co., Grand Island, New York).
Island, N. Y.) at pH 7.2. The trachea was occluded, and the lungs were suspended in a glutaraldehyde-filled beaker for 1 hr at room temperature. The lungs were then cut with a scalpel into pieces no larger than 12 x 6 x 2 mm. Tissue samples were cut from the trachea, from each lobe of the lungs in a plane including a longitudinal section of the major bronchus, and from areas suspected of pathology. At least 6 samples were thus taken from each animal. The tissue samples were dehydrated, infiltrated in a desiccator with hematoxylin-phloxine and PAS3-hematoxylin, as has been previously described (10). For paraffin sections the inflamed lungs and trachea were removed together, fixed in alcohol-formalin fixative, and embedded in paraffin blocks. Sections 5-μm thick were cut and stained routinely with hematoxylin and eosin, Verhoeff-Van Gieson, and PAS-hematoxylin.

RESULTS

Analysis of the plastic-section slides from the serial-sacrifice 210Po-0.9% NaCl group revealed the following sequential changes in the pathogenesis of the tumors. The earliest change observed, by 7 days after the second instillation of 210Po, was hyperplasia and hypertrophy of type 2 alveolar cells. Such hyperplastic type 2 alveolar cells containing an increased number of cytoplasmic inclusions or cytosomes are shown in Fig. 1. Focal areas of inflammation were observed throughout the instillation period but were observed only occasionally at later times. These areas showed edema, polymorphonuclear leukocytes, and mononuclear cells.

At 7 days after the fifth instillation of 210Po, the first example of "alveolar epithelialization or bronchiolization" was seen. This is a descriptive term applied to the appearance of bronchiolar epithelial cells lining the surface of preexisting alveoli where normally only alveolar-type cells occur. Both ciliated and typical bronchiolar Clara cells appear in these alveolar lesions (15, 27). Early "epithelialization" is shown in Fig. 2, in which the atypical cells appear to arise in an otherwise normal lung and do not appear to be connected with a bronchiole. A more advanced region, in which the degree of epithelialization gives the appearance of "hyperplastic nodules," is shown adjacent to a bronchiole in Figs. 3 and 4. Such hyperplastic nodules were most often seen near bronchioles in the lung sections. Ciliated cells were seen less frequently in these areas; as the pathological regions containing acinar structures enlarged, ciliated cells were replaced by the proliferation of Clara-type cells. Lung macrophages were often seen within hyperplastic nodules, as shown in Figs. 3 and 4.

By 17 weeks after the first instillation of 210Po, the hyperplastic nodules contained cells which resembled Clara cells but secreted a PAS-positive product, shown in Fig. 5. Both single- and double-layered nodules containing PAS-positive material were observed. Some of the hyperplastic nodules contained very flattened cells, as shown in Fig. 6. These resembled squamous cells and also secreted PAS-positive material. It was not clear whether these "squamous" cells were derived from hyperplastic Clara cells, but the PAS-positive product secreted is similar (15). Many Clara cells of the normal bronchiolar epithelium, not normally PAS positive, also began to display PAS-positive granules similar to those seen in cells of the hyperplastic regions. Clara cell hyperplasia in bronchioles was also observed frequently after 17 weeks. The Clara cells were larger than normal and often contained 2 nuclei with prominent multiple nucleoli.

Tumors classified as combined epidermoid and adenocarcinomas, shown in Figs. 7 to 11, appeared to arise within regions of hyperplastic nodules. Frankly malignant tumors showed varying cellular atypia with mucus production nearly always present in some areas of the tumor. These tumors destroyed the supporting lung stroma and invaded airways, blood vessels, and pleura, as shown in Figs. 8 to 11. What we have classified as a borderline tumor is a hyperplastic lesion that does not have the invasive characteristics observed in the malignant tumors; characteristics of borderline tumors have been discussed in more detail elsewhere (24).

The first tumor classified as cancer appeared in the 210Po-0.9% NaCl plastic section series 22 weeks after the first instillation of 210Po. In the other series studied, pathological changes appeared at approximately the same times, with the earliest malignant tumor occurring at 15 weeks in the 210Po-ferric oxide paraffin section serial-sacrifice group. The latency period appeared to be shorter for the 210Po-ferric oxide serially sacrificed animals than it was for the 210Po-0.9% NaCl animals. In Chart 1 the cumulative percentage of tumor-bearing animals is graphed against time. Final tumor incidence data for each group are shown in Table 1. Of the 67 animals bearing malignant tumors, 13 had metastatic tumor cells within the pleural cavity, in the walls of blood vessels or lymphatics. No distant metastases were found.

As we believe there is a progression from the earliest epithelialization of alveoli to malignant tumors, hyperplasia of bronchiolar-type cells in the alveolar region is considered a premalignant lesion. In an effort to quantify such pre malignant changes, all of the lung tissue included in the

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* The abbreviation used is: PAS, periodic acid-Schiff.

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Chart 1. Cumulative percentage of tumor-bearing animals versus time. O, animals receiving 210Po-ferric oxide instillations; x, animals receiving 210Po-0.9% NaCl. Data were collected from serially sacrificed animals in both 210Po treatment groups.
Animals bearing tumors frequently had more severe hyperplasia than did non-tumor-bearing animals sacrificed at similar times. As shown in Chart 2, tumor-bearing animals always had hyperplasia scores of 4 or greater, while non-tumor-bearing animals frequently had scores of less than 4. This phenomenon was also observed for the dead animals in both 210Po treatment groups. The extent and histopathological characteristics of hyperplastic nodules observed at late sacrifice times were comparable to those seen at earlier sacrifice times.

The histopathological characteristics of the tumors did not change with time, in that the epidermoid component was equally prominent at early and late sacrifice times. The tumors described as early as 15 to 25 weeks after the first instillation of 210Po appeared equally as aggressive (in terms of histopathological characteristics and invasion) as did those tumors observed at later times. Metastatic tumor cells were, however, observed somewhat more frequently at the

### Table 1

**Tumor incidence in hamsters exposed to 210Po**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of tumors</th>
<th>No. of tumor-bearing animals/ no. of animals in group</th>
<th>Total tumor incidencea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>1. 210Po-0.9% NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Plastic section serials</td>
<td>10</td>
<td>13</td>
<td>23/63 (36.5)</td>
</tr>
<tr>
<td>b. Paraffin section serials</td>
<td>9</td>
<td>10</td>
<td>19/52 (36.5)</td>
</tr>
<tr>
<td>c. Dead</td>
<td>9</td>
<td>14</td>
<td>23/60 (38.3)</td>
</tr>
<tr>
<td>2. 210Po-ferric oxide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Plastic section serials</td>
<td>12</td>
<td>12</td>
<td>24/59 (40.1)</td>
</tr>
<tr>
<td>b. Paraffin section serials</td>
<td>10</td>
<td>11</td>
<td>21/56 (37.5)</td>
</tr>
<tr>
<td>c. Dead</td>
<td>17</td>
<td>10</td>
<td>27/85 (31.8)</td>
</tr>
</tbody>
</table>

a These incidence figures include animals that died or were sacrificed during the instillation period. The tumor incidence among animals dying or sacrificed after the instillation period is as follows: Group 1, a and b = 42 of 101 (41.6%); Group 1, all animals = 65 of 155 (41.9%); Group 2, a and b = 45 of 101 (44.6%); Group 2, all animals = 72 of 179 (40.2%).

b Numbers in parentheses, percentage.

For each animal a single number was used to describe the degree of hyperplasia of bronchiolar-type cells in the alveolar region. This number was obtained by multiplying the number given for intensity of hyperplasia and the number given for extent (or multifocality) of hyperplasia. The data obtained from the lung of each animal in the serially sacrificed groups and analyzed in this fashion have been graphed in Chart 2. In addition, animals with borderline or malignant tumors are indicated. Thus, tumor-bearing animals appear twice in this chart.

Hyperplastic changes were more intense and frequent at earlier times in the 210Po-ferric oxide animals than in the 210Po-0.9% NaCl animals, which is consistent with the difference in the latency period for tumor development in the 2 treatment groups. In each group studied the lesions progressed very rapidly, within just a few weeks, from minimal hyperplastic changes to malignant tumors (Chart 2). There was also much variation from animal to animal within each group. At practically every time studied beyond 18 weeks in both the 210Po-0.9% NaCl and 210Po-ferric oxide groups, there were animals bearing malignant lung tumors as well as other animals bearing minimal hyperplastic changes or no microscopically abnormal regions at all. Animals bearing tumors frequently had more severe hyperplastic changes than did non-tumor-bearing animals sacrificed at similar times. As shown in Chart 2, tumor-bearing animals always had hyperplasia scores of 4 or greater, while non-tumor-bearing animals frequently had scores of less

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later sacrifice times. In general, larger tumors were found at later times. $^{210}$Po-induced tumors are multifocal in origin; animals bearing tumors in this study frequently had several borderline or malignant tumors. The tumors observed in the animals that died during the experiment were not larger or more aggressive in character than were those observed in the serially sacrificed animals, and the time courses of hyperplasia and tumor development in the dead animals were similar to those of sacrificed animals in each treatment group. One difference between the sacrificed and dead animals is the degree of acute inflammation. Inflammatory changes were widespread in the dead animals but were observed only occasionally in the sacrificed animals. Many of these hamsters may have died of pneumonia.

**DISCUSSION**

The abnormalities observed in the peripheral lung are well correlated with the distribution of radioactivity following either $^{210}$Po-0.9% NaCl or $^{210}$Po-ferric oxide intrathoracic administration (11, 12, 17). In the animals receiving $^{210}$Po-ferric oxide, pathological changes were observed primarily around collections of ferric oxide particles within lung macrophages in the respiratory bronchiolo-alveolar duct regions of the lung (11, 12). $^{210}$Po remains firmly bound to the ferric oxide carrier particles (12, 22); the distribution and movement of ferric oxide particles in the lung with time has been previously described (12, 35).

The final tumors induced by $^{210}$Po-ferric oxide or $^{210}$Po-0.9% NaCl were identical, as were the premalignant changes. The morphology of the $^{210}$Po-induced tumors did not change with time. Tumors observed at both early and late sacrifice times showed the combined epidermoid and adenocarcinomatous morphology. These tumors are primarily adenocarcinomas in which varying amounts of squamous metaplasia has occurred. The squamous metaplasia is considered “pseudoepidermoid,” in that it is not a keratinizing epithelium (15, 24). It is probable that these areas are derived from atypical Clara cells, since the secretion product is similar (15). Although there are cells within these tumors that have obvious epidermoid characteristics at the electron microscopic level (15), the adenomatous component predominates when these tumors are transplanted (18, 36).

The first change observed in the lungs of $^{210}$Po-exposed hamsters was hypertrophy and hyperplasia of type 2 alveolar cells. This response has been observed after a variety of injuries to the lung, including chronic irritation (34), nitrogen dioxide exposure (38), and drugs (37). Although atypical type 2 alveolar cells are found within and at the edges of $^{210}$Po-induced tumors, they are not thought to be involved in tumor pathogenesis (15).

Subsequently, there appears to be a rapid progression from early hyperplastic changes to more severe and extensive hyperplasia and then to borderline and malignant tumors that grow larger with time. Thus, $^{210}$Po-induced lung tumors develop in a stepwise progression, as has been shown previously for carcinogenesis in other systems (5). Epithelialization or bronchiolization of alveoli (27) is probably the first stage in this spectrum of responses leading to tumors. This condition, in which bronchiolar-type cells line the preexisting alveolar walls, has also been observed after many unrelated injuries to the lung (27), including exposure to viruses (18, 26) and chemical carcinogens (31). Areas of epithelialization progress to what we have termed hyperplastic nodules, in which primarily Clara-like cells form acinar structures in the lung. We consider hyperplastic nodules a definite precursor lesion in tumor formation (15). It is not clear whether these hyperplastic nodules in the lung are reversible. At a later stage hyperplastic nodules secrete PAS-positive materials, primarily neutral mucopolysaccharides and a neuraminidase-resistant sialomucin (15). Squamous metaplasia of epithelialized alveolar tissue, a later event in tumor development, is thought to be a reversible lesion that can be observed after chronic irritation (8, 20) or exposure to other carcinogens such as $^{106}$Ru (20), polycyclic hydrocarbons (30), and oncogenic viruses (18). Squamous metaplasia and the appearance of mucous-secreting cells in the alveoli are also considered early changes in the pathogenesis of squamous cell cancer in the peripheral lung induced by pathogenic viruses (18).

Although inflammatory changes have been previously implicated in the induction of lung cancer from radiation (1, 32), there is no evidence from this study that inflammation is an important part of the process. Interestingly, widespread inflammatory changes were frequent in animals that died spontaneously but were rarely seen in sacrificed animals. By examining dead or dying animals only, one might gain the erroneous impression that tumor induction in hamsters is associated with inflammatory changes in the lung. Similarly, inflammatory changes do not appear to be necessary for the development of lung cancer from chronic $\beta$-radiation of the lung (9) or low doses of inhaled $^{210}$Po (39). Inflammation may accelerate the carcinogenic process, perhaps by the stimulation of cell proliferation. The role of inflammation in lung carcinogenesis has been discussed previously (14).

Although the final tumor incidence was approximately the same in the 2 treatment groups, hyperplastic changes and tumors appeared at earlier times in the $^{210}$Po-ferric oxide serial-sacrifice groups than they did in the $^{210}$Po-0.9% NaCl animals. This difference in latency period could be due to the greater dose received by the $^{210}$Po-ferric oxide animals (2700 rads) compared to the $^{210}$Po-0.9% NaCl animals (1000 rads). Larger doses of radiation result in shorter latent periods and a higher tumor incidence (14, 22-24), although data from previous experiments indicate that tumor incidence should be similar for the dose range of $^{210}$Po-0.9% NaCl and $^{210}$Po-ferric oxide studied here (22, 24). The final tumor incidence figures reported in this study are slightly lower than are those previously reported for similar doses (22, 24) because of the inclusion of those animals that died or were sacrificed during the instillation period. Such “early deaths” have not been included in previous tumor incidence data (24).

Clearly, some animals are very resistant to the induction of lung cancer from $^{210}$Po, as has been shown for other lung carcinogens (1, 14). At every sacrifice time studied in both treatment groups after 18 weeks, there were animals with no abnormality as well as animals with severe hyperplasia and tumors; in general the animals that developed tumors also showed more severe hyperplastic changes in
other parts of the lungs, as seen in Chart 2. It has been observed that animals dying late after substantial doses of 210Po by intratracheal instillation are often free of lung tumors (22). In this experiment many of the animals in the 210Po-ferric oxide group that died after 70 weeks (9 of 14, 64%) had neither lung tumors nor areas of focal hyperplasia. In the 210Po-0.9% NaCl group, only 4 animals died after 70 weeks; 3 of these animals had lung tumors, while 1 animal had a normal lung.

In general the animals that died during the course of the experiment showed the same variations in lung pathology and the same time course of tumor development as those observed in the sacrificed animals. As has been previously concluded (24), animals appear to be dying with, rather than of, these 210Po-induced peripheral lung tumors. Such tumors in rodents do not metastasize as readily as do similar tumors in other species (7, 28); in this study only 13 cases of tumor metastases were found.

Although the malignant tumors appeared to increase in size with time, severe hyperplasia and small tumors also appeared at late sacrifice times in this study. A probable explanation for this is different growth rates for these tumors. Those appearing late in life may grow more slowly than those appearing at the early sacrifice times. Another explanation is that tumors can be initiated late in the hamster life span, since some of the 210Po is retained in the lung for a long period. However, the dose rate is very low at late sacrifice times; most of the 210Po is cleared rapidly from the lung following intratracheal instillation (11, 12, 17).

Separate radiochemical studies have shown that, following 7 intratracheal instillations of 210Po-0.9% NaCl or 210Po-ferric oxide in the dose range studied here, approximately 10% of the weekly administered 210Po-ferric oxide dose and 1% of the 210Po-0.9% NaCl dose are retained in the lungs 38 weeks after the first 210Po instillation (A. R. Kennedy and J. B. Little, unpublished data). The appearance of late hyperplastic nodules suggests that some of these lesions may not go on to form true tumors, at least within the life span of the animal.

ACKNOWLEDGMENTS

We would like to thank Frank Bettinelli for his expert assistance in the preparation of histological materials.

REFERENCES

Fig. 1. An early response of the lung to $^{210}$Po exposure: hypertrophy and hyperplasia of type 2 alveolar cells. These atypical type 2 alveolar cells contain many more cytosomes than are usually found. Ten weeks after the first $^{210}$Po-0.9% NaCl instillation. Plastic section, PAS-hematoxylin, x 500.

Fig. 2. Epithelialization of alveoli (bottom left and right) occurring within otherwise normal lung tissue. Seven weeks after the first $^{210}$Po-0.9% NaCl instillation. Plastic section, hematoxylin-phloxine, x 40.

Fig. 3. A more advanced area of epithelialization of alveoli in which hyperplastic nodules have formed (left). Right, normal bronchiole. Sixteen weeks after the first $^{210}$Po-0.9% NaCl instillation. Plastic section, PAS-hematoxylin, x 125.

Fig. 4. A higher-power view of the hyperplastic nodules shown in Fig. 3. Normal bronchiolar epithelium appears at right. The epithelium of the hyperplastic nodules contains typical bronchiolar Clara and ciliated cells. Typical lung macrophages are seen trapped within these nodules. x 250.

Fig. 5. An advanced hyperplastic nodule containing 2 layers of cells. PAS-positive mucous secretions in center of acinar structure and in the apical region of the epithelial cells. Twenty-six weeks after the first instillation of $^{210}$Po-0.9% NaCl. Plastic section, PAS-hematoxylin, x 500.

Fig. 6. An area of squamous metaplasia appearing within a $^{210}$Po-induced tumor. Twenty-six weeks after the first instillation of $^{210}$Po-0.9% NaCl. Plastic section, hematoxylin-phloxine, x 250.
Fig. 7. Combined epidermoid and adenocarcinoma showing bronchiolar invasion. No variability in morphology of these tumors from field to field. An adenomatous area (left) shows PAS-positive mucous secretions, while secretions are not present at right. Twenty-six weeks after first instillation of $^{111}$Po-ferric oxide. Plastic section, PAS-hematoxylin, × 225.

Fig. 8. Lung tumor with extension into pleural cavity. Arrow, pleural elastica. The presence of tumor cells outside the pleura (left) is clear evidence of invasion into the pleural cavity. Twenty-four weeks after the first instillation of $^{111}$Po-0.9% NaCl. Paraffin section, Verhoeff-Van Giesen, × 500.

Fig. 9. Blood vessel with subintimal metastatic combined epidermoid and adenocarcinoma. Sixty-one weeks after the first $^{111}$Po-ferric oxide instillation. Plastic section, PAS-hematoxylin, × 450.
Fig. 10. Lung section showing combined epidermoid and adenocarcinoma (center bottom) and focus of metastatic or invasive tumor in wall of a pulmonary artery (center left). There is also an incidental benign papilloma (top right) within the bronchus. Sixty-one weeks after the first $^{210}$Po-ferric oxide instillation. Plastic section, PAS-hematoxylin, × 225.

Fig. 11. Higher-power view of Fig. 10 showing invasive or metastatic tumor cells in artery wall. × 900.
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