Primary Tumors of Bone and Lung in Rats Following Local Deposition of Cupric-chelated N-Hydroxy-2-acetylaminofluorene

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SUMMARY

The deposition of 14.2 mg of cupric-chelated N-hydroxy-2-acetylaminofluorene in the intramedullary cavity of the right femur and 12.6 mg of nonchelated N-hydroxy-2-acetylaminofluorene in the intramedullary cavity of the left femur of 10- to 16-week-old female Osborn-Mendel rats resulted in embolism of the compounds to the lungs in 12 of 32 rats and the development of epidermoid neoplasms in the lungs of 3 of 29 rats that survived. Twenty-eight sarcomas developed at the site of injection of the cupric chelate in 21 of the 29 rats during an observation period of 55 weeks; only one rat developed sarcomas at the site of injection of the nonchelated compound. Three of the 28 tumors were rhabdomyosarcomas; an additional 3 giant-cell sarcomas were presumed to be extraosseous in origin. Twenty-two tumors in 17 rats were composed of either bone-forming or bone marrow elements. Nine of the tumors were predominantly of osteogenic cells, two of chondrogenic cells, one of fibrogenic cells, four of reticulum cells, two of plasma cells, and four of vasoformative tissues. However, many of these tumors contained more than one type of neoplastic cell. Of note were four tumors composed of mixtures of reticular tissues and three tumors composed of both osteogenic and vasoformative neoplastic cells. These complexities of structure and behavior simulated well the characteristics of the bone tumors of man.

INTRODUCTION

During an experiment to determine the response of pulmonary tissues of the rat to N-hydroxy-2-acetylaminofluorene (N-HO-AAF),1 an osteosarcoma of the rib formed at the site of an intrathoracic pellet that contained the cupric chelate [Cu(N-HO-AAF)]2 of this compound (19). The following experiment was carried out to determine the response to deposition of each of these compounds in the intramedullary cavity of the femur of the rat.

MATERIALS AND METHODS

Drs. Elizabeth and John Weisberger (National Cancer Institute) provided the N-HO-AAF and the copper chelate of this compound, prepared according to methods previously described (15, 22, 16). Both compounds were stored in opaque containers at 4°C until used. The chemicals, finely ground, were prepared for injection by adding them to a vehicle consisting of 4 parts tricaprylin to 1 part purified USP beeswax. Experience with this vehicle during the past four years has indicated that it is not carcinogenic in rats. An effort to obtain mixtures of equimolar concentrations of N-HO-AAF was made by mixing the cupric-chelated compound at a level of 283 mg/ml of vehicle and the nonchelated compound at a level of 253 mg/ml of vehicle. After brief heating at 70°C, the liquefied vehicle was mixed with each of the compounds and drawn into 1-ml syringes. The suspensions were continuously shaken until they cooled to room temperature to form a pasty mixture that could be injected through a 19-gauge needle.

The intramedullary cavities of both femurs were injected with 0.05 ml of the mixtures. The right femur received approximately 14.2 mg of the chelated compound, and after a recovery period of two weeks the left femur received approximately 12.6 mg of the nonchelated compound. The method of injection was new to us, although we later found reference to similar methods (7, 9). Under ether anesthesia, the knee joint was shaved, scrubbed, and incised. The patellar ligament was severed just proximal to the patella and reflected downward, exposing the intercondylar surface of the femur. This surface was penetrated with a No. 5 round dental burr, and a 1½-inch, 19-gauge needle was passed several times through the length of the marrow cavity. A second 19-gauge needle attached to the syringe containing the mixture was then passed into the femoral marrow cavity and the mixture deposited slowly as the needle was withdrawn. Care was taken to remove the mixture from the articular surface, but the hole in the bone was not plugged. The patellar ligament and skin were closed separately with cotton sutures. Healing in all was rapid and without severe infection.

Thirty-two female rats, 10 to 16 weeks old, from the closed colony of the Animal Production Section, NIH, were treated. The rats were derived from Osborn-Mendel strain stock, but they were not inbred. Three rats died within 24 hours after injection. Of the remaining 29, 19, including 13 with grossly apparent tumors, were killed between 21 and 32 weeks; the remaining 10 were killed between 40 and 55 weeks after injection. Since the rats were not inbred, no attempt was made to transplant the tumors. After fixation in 10% formalin, histologic sections were made from both hind legs, the lungs, and organs showing gross abnormalities. Representative soft-tissue tumors and longitudinal step-sections of both hind legs, decalcified in

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1 Chemical Abstracts nomenclature: N-2-fluorenylacetoxyhydroxamic acid.

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TABLE 1
Sarcomas of the Leg in Female Strain OM Rats following Intrafemoral Injection of 14.2 mg Cupric-Chelated N-Hydroxy-2-acetylamino-Fluorene

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Week killed</th>
<th>Tumor (secondary tumor, cell types)*</th>
<th>Tumor site</th>
<th>Tumor size (mm)b</th>
<th>Degree of tumor cell dysplasia</th>
<th>Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>24</td>
<td>Fibrosarcoma</td>
<td>Distal metaphysis</td>
<td>25 +</td>
<td>+++</td>
<td>Lung, nodes</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>Reticulum cell sarcoma (Myeloblast, macrophage)</td>
<td>Proximal diaphysis</td>
<td>70 +</td>
<td>++</td>
<td>Lung, nodes</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>Rhabdomyosarcoma (Fibroblast)</td>
<td>Extraskeletal</td>
<td>80 +</td>
<td>++</td>
<td>Lung</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>Plasma cell myeloma (Fibroblast)</td>
<td>Distal diaphysis</td>
<td>80 +</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>25</td>
<td>Reticulum cell sarcoma (Plasma cell)</td>
<td>Proximal diaphysis</td>
<td>65 +</td>
<td>+</td>
<td>Viscera</td>
</tr>
<tr>
<td>12</td>
<td>25</td>
<td>Osteogenic sarcoma (Fibroblast)</td>
<td>Distal diaphysis</td>
<td>15 +</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>25</td>
<td>Rhabdomyosarcoma (Fibroblast)</td>
<td>Extraskeletal</td>
<td>30 +</td>
<td>+++</td>
<td>Viscera</td>
</tr>
<tr>
<td>15</td>
<td>25</td>
<td>Fibrosarcoma (Giant cells)</td>
<td>Extraskeletal</td>
<td>90 +</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>30</td>
<td>Osteogenic sarcoma (Chondroblast, giant cell)</td>
<td>Proximal periosteum</td>
<td>60 +</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>31</td>
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<td>Extraskeletal</td>
<td>10 +</td>
<td>++</td>
<td>Lung</td>
</tr>
<tr>
<td>19</td>
<td>32</td>
<td>Fibrosarcoma (Giant cell)</td>
<td>Extraskeletal</td>
<td>15 +</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>32</td>
<td>Hemangiosarcoma (mixed) (Chondroblast, osteoblast)</td>
<td>Proximal diaphysis</td>
<td>25 +</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>32</td>
<td>Hemangiosarcoma (Fibroblast)</td>
<td>Distal diaphysis</td>
<td>35 +</td>
<td>+++</td>
<td>Lung</td>
</tr>
<tr>
<td>21</td>
<td>32</td>
<td>Osteogenic sarcoma (Fibroblast)</td>
<td>Mid-diaphysis</td>
<td>20</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>32</td>
<td>Rhabdomyosarcoma (Osteoblast)</td>
<td>Extraskeletal</td>
<td>40 +</td>
<td>+++</td>
<td>Lung</td>
</tr>
<tr>
<td>23</td>
<td>40</td>
<td>Hemangiosarcoma (mixed) (Chondroblast, osteoblast)</td>
<td>Distal metaphysis</td>
<td>40 +</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>40</td>
<td>Osteogenic sarcoma</td>
<td>Proximal epiphysis</td>
<td>15 +</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>44</td>
<td>Osteogenic sarcoma</td>
<td>Proximal periosteum</td>
<td>10 +</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>44</td>
<td>Hemangiosarcoma</td>
<td>Distal metaphysis</td>
<td>10 +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>49</td>
<td>Osteogenic sarcoma</td>
<td>Proximal metaphysis</td>
<td>15 +</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>49</td>
<td>Reticulum cell sarcoma (Plasma cell, osteoblast?)</td>
<td>Distal femur</td>
<td>65 +</td>
<td>++</td>
<td>Lung</td>
</tr>
<tr>
<td>27</td>
<td>50</td>
<td>Osteogenic sarcoma (mixed) (Angioblast, fibroblast)</td>
<td>Mid-diaphysis</td>
<td>35 +</td>
<td>+++</td>
<td>Lung</td>
</tr>
<tr>
<td>27</td>
<td>50</td>
<td>Plasma cell myeloma</td>
<td>Distal metaphysis</td>
<td>&lt;5 +</td>
<td>+</td>
<td>Lung</td>
</tr>
<tr>
<td>29</td>
<td>55</td>
<td>Reticulum cell sarcoma (Fibroblast, osteoblast?)</td>
<td>Distal epiphysis</td>
<td>&lt;5 +</td>
<td>+</td>
<td>Lung</td>
</tr>
<tr>
<td>30</td>
<td>55</td>
<td>Osteogenic sarcoma</td>
<td>Distal periosteum</td>
<td>45 +</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>55</td>
<td>Chondrosarcoma (Osteoblast)</td>
<td>Proximal metaphysis</td>
<td>20 +</td>
<td>+++</td>
<td>Lung</td>
</tr>
<tr>
<td>31</td>
<td>55</td>
<td>Osteogenic sarcoma</td>
<td>Distal diaphysis</td>
<td>10</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* Many of the tumors possessed more than one type of cell. These cell types are listed only when they appeared to be neoplastic and not the result of reactive inflammation or repair.

b +, local invasion.
formic-sodium citrate solution, were stained routinely with hematoxylin-eosin and selectively with phosphotungstic acid-hematoxylin, periodic acid-Schiff's stain, Masson's trichrome, Mallory-Heidenhain Azan anilin blue, von Kossa's stain for calcium, and Wilder's stain for reticulum. Fixed frozen sections of lungs were stained with oil red 0 for evidence of fat embolism. Femurs from 16-, 35-, and 65-week-old untreated rats were studied by similar methods.

RESULTS

The three rats that died in the first 24 hours after injection indicated the hazard of intramedullary injection. All three had emboli of olive-green, anisotropic crystals of Cu(N-HO-AAF)₂ in both the inferior vena cava and main branches of the pulmonary artery. Subsequently, similar material and fat emboli were found in the lungs of 9 of the 29 survivors. Two of the 9 had keratinizing epidermoid cysts, and 1 had an invasive epidermoid carcinoma of the lung, all adjacent to emboli of Cu(N-HO-AAF)₂. Details of these pulmonary tumors will be published separately with the results of other pulmonary carcinogens.

In the 3 rats that died early, the needle tracts were filled entirely with carcinogen, some being extruded onto the articular surface (Fig. 1). Primary reaction to the two compounds was similar in rats killed subsequently. The involved marrow was replaced by loose connective tissue that contained foreign body granulomas. The affected intramedullary canal was narrowed and walled off from adjacent marrow by thickened, remodeled trabecular bone, and persistent periosteal osteogenesis was present in the overlying cortex (Fig. 2). Crystals of Cu(N-HO-AAF)₂ were the only particles that could be identified, and these persisted either in the intramedullary canal or, when the bone was destroyed by tumor, in the adjacent soft tissues through 55 weeks of the experiment.

Only one rat had tumors in the leg injected with N-HO-AAF. This rat (No. 31, Table 1), killed at 55 weeks, had both a low-grade intramedullary osteogenic sarcoma that involved the distal metaphysis and, separate from this tumor, a poorly differentiated reticulum cell sarcoma with myeloblastic elements that involved the entire proximal end of the femur (Figs. 3, 6).

Twenty-one of the 29 survivors had sarcomas in the leg injected with Cu(N-HO-AAF)₂. Seven of the 21 had two distinctly separable sarcomas in this leg. The 28 sarcomas are listed in Table 1 according to the most reasonable diagnosis and site of origin; however, this grouping was far from absolute for all of the tumors. Six of the tumors were considered extrasosseous in origin, three were rhabdomyosarcomas, and three were fibrosarcomas. The latter were characterized by giant-cell formation (Fig. 9) and by marked osteoblastic activity where they merged with bone. All three could have been derived from the periosteum or from the soft tissues of the joint, but no unequivocal evidence for such origin could be found.

The remaining 22 tumors were derived from tissues of the bone, but classification was not always simple. On the basis of the predominant cell type, nine of the tumors were classified as osteogenic sarcomas and two as chondrogenic sarcomas (Figs. 7, 8), but a generous mixture of the two types of cells occurred in four of the tumors in this group (Fig. 4). The periosteum was the obvious source of three of the osteogenic sarcomas whereas three others remained confined to the intramedullary canal, attesting to their endosteal origin. No topographic predilection for chondrogenic versus osteogenic activity was apparent, but the series was small.

Nine tumors were composed exclusively of tissue elements of the bone marrow. One was an intramedullary fibrosarcoma that metastasized early and widely. Two tumors at both extremes of size were solitary plasma cell myelomas, being composed of osteolytic masses of uniform "cart-wheel" cells of the type in Fig. 12. The larger of the two contained areas of amyloid-like ground substance. Two tumors were hemangiosarcomas composed exclusively of large vascular channels lined by thick layers of neoplastic endothelium (Fig. 10). Lung metastases in one tumor retained clearly identifiable vascular characteristics. The remaining four tumors derived from marrow elements were classified as reticulum cell sarcomas because of their predominant tissue characteristics, but areas of either myeloblastic cells, plasma cells, or cells that were entirely undifferentiated could be found in all. The undifferentiated cells were small uniform cells with poorly defined cytoplasm and without supporting reticulum and resembled Ewing's sarcoma of man. A separate classification did not seem justified since partially differentiated reticular tissues of one type or another could be found in all, but the study of further tumors of this type may offer clues to the histogenesis of Ewing's sarcoma.

Aside from the mixing of reticular tissues in some tumors and osteogenic and chondrogenic tissues in others, the three remaining tumors had an even more complex composition. Rats #19, 23, and 27 each had invasive sarcomas in which the predominant component was vascular, having the same structure as the hemangiosarcoma that metastasized in Rat #20 (Fig. 10). However, in all three tumors the vascular components merged with areas of highly dysplastic chondrogenic and osteogenic tissue (Fig. 5). The possibility that the latter represented atypical, reactive callus formation was considered; however, in Rat #27 pulmonary metastases from the mixed tumor were entirely of osteogenic cells.

DISCUSSION

Although the development of osteogenic sarcoma after radium poisoning is a classic example of cancer induction in man (14), the induction of bone tumors in animals has rarely been accomplished. A directional review in 1953 notes that bone tumors have been induced in rodents with salts of radium, mesothorium, and beryllium, with externally applied X-rays, and rarely with compounds of nickel, chromium, cobalt, and the more conventional carcinogens, 3-methylcholanthrene, 7,12-dimethylbenz(a)anthracene, and benzo(a)pyrene (21). More recently, the
radionuclides, strontium-89, strontium-90, calcium-45, yttrium-90, phosphorus-32, and plutonium-239 have been found to be effective (2, 3, 13). With the possible exception of bone tumor induction by beryllium salts and the radionuclides, no convenient model has been found for experimentation because beryllium is capable of inducing bone tumors only in rabbits and the radioactive compounds are difficult materials to handle.

Bone tumors have also been induced in mice, rats, and chickens by onogenic viruses (1, 4, 11, 12, 18). Our impression of the onogenic tumors induced by polyoma virus is that they represent neoplasia in its most primitive form. Characteristically, the tumor cells are distinguished in structure by their uniform immaturity and in behavior by an exceptional lack of aggressiveness. Initial growth seems to be simply the result of inadequately controlled focal osteogenesis (20). Contrary to one report (5), progression in either direction may occur and the onogenic tumors occasionally metastasize, but most of the tumors have evidence of cessation of growth and end as sessile osteophytic masses. The proliferative lesions of the bone induced by the avian leukosis virus complex also seem to represent a simple defect in growth control (6, 11, 17).

The bone tumors induced with Cu(N-HO-AAF), in this experiment seem to represent the opposite extreme in neoplastic development to that of the viral-induced tumors. Not only are the chemically induced tumors consistently invasive and capable of metastasizing but also many different tissue components are represented in the tumors, and diversities in cell type occur even in the same tumor. The latter apparent deviations in differentiation are common phenomena in the bone tumors of man. Such mixed tumors may form by coalescence of separate neoplastic foci. In the present experiment this does not seem unlikely, but it is also possible that the variations in differentiation represent progeny of a single cell clone that have taken different pathways. It should be noted that, unlike most viruses, the cupric-chelated carcinogen remains as a carcinogen in the tissues over a long period of time. This persistence may account for the diverse character of the tumors, the carcinogen acting continuously on either a number of different cell clones or the progeny of cells already neoplastic. Several observers maintain that, in man, bone tumors may be derived from a totipotential cell that differentiates along either bone-forming or blood-vascular lines, depending on environmental factors (8, 10). Perhaps the two contrasting models of bone oncogenesis will help resolve these and other questions.

ACKNOWLEDGMENTS

Mr. Joseph Albrecht and staff were responsible for the histology preparations and Mr. Gebhard Gsell for the photographs.

REFERENCES


Fig. 1. Carcinogen in intrafemoral canal 48 hours after deposition. Rat from recent experiment in which trephine hole in articular surface was plugged with beeswax. Note absence of carcinogen and wedge-shaped configuration of the plug in the epiphysis. H & E, × 11.

Fig. 2. Primary reaction to cupric-chelated N-hydroxy-2-acetylaminofluorene in the diaphysis. Note granulomatosis of the affected marrow and walling-off by osteosclerotic trabeculae and thickened cortical bone. H & E, × 15.

Fig. 3. Low-grade intramedullary osteogenic sarcoma involving the distal metaphysis of the left femur of Rat #31, treated with N-hydroxy-2-acetylaminofluorene. Note the thick remodeled cortical bone characteristic of the primary reaction to the compounds. H & E, × 25.

Fig. 4. Segment of sarcoma of proximal metaphysis in Rat #26. In the intramedullary canal the tumor forms bone, but outside the femur only cartilage is formed. H & E, × 16.

Fig. 5. Mixed tumor of the diaphysis in Rat #19. Central area has structure similar to Fig. 10. On the right the tumor is entirely cartilagenous; other areas were osteogenic sarcoma. H & E, × 11.

Fig. 6. Detail of myeloblastic area of a massive reticulum cell sarcoma predominantly like that of Fig. 11. Tumor from Rat #31, proximal end, left femur, treated with N-hydroxy-2-acetylaminofluorene. H & E, × 520.

Fig. 7. Chondrogenic sarcoma of the type observed in areas of bone-forming tumors in six rats. H & E, × 135.

Fig. 8. Poorly differentiated osteogenic sarcoma of the type observed in areas of bone-forming tumors in thirteen rats. H & E, × 210.

Figs. 9A–B. Giant-cell sarcoma of the type observed in 3 rats developing outside the bone. H & E, Fig. 9A × 135, Fig. 9B × 380.

Figs. 10A–B. Hemangiosarcoma of the type observed in 5 rats. H & E, Fig. 10A × 85, Fig. 10B × 280.

Figs. 11A–B. Reticulum cell sarcoma of the type observed in 4 rats. H & E, Fig. 11A × 125, Fig. 11B × 290.

Figs. 12A–B. Plasma cell myeloma of the type observed in 4 rats. Note amyloid-like ground substance in lower left. H & E, Fig. 12A × 135, Fig. 12B × 380.
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