Actinomycin D: Effects on Ridgway Osteogenic Sarcoma in Mice

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Summary

Destruction of Ridgway osteogenic sarcoma (ROS) tumor in AKR mice occurs after single i.v. injections of actinomycin D. Optimal doses for full regression of the tumor were 800–1200 \( \mu g/kg \). Doses of 400 \( \mu g/kg \) were only partially effective and those of 1600 \( \mu g/kg \) were lethal. The therapeutic response probably occurs without assistance by host immune mechanisms. This was demonstrated by the successful growth of 2nd implants in animals in which primary tumors were regressing after treatment by the agent. When actinomycin was given by i.p. route, mice were lethally intoxicated by 1200 \( \mu g/kg \), even though this dose was tolerated when administered by i.v. route. Tissues were taken from tumor-bearing mice for pathologic examination at various times after i.v. and i.p. administration of actinomycin D (800 \( \mu g \)). Recovery of nucleated elements in spleen of mice treated i.p. was markedly delayed by comparison with that found after i.v. doses. Other tissues (e.g., small intestine, thymus, and liver) as well as tumor were not differentially affected by the 2 routes. Twenty-four hr after treatment (800 \( \mu g/kg \) by either route), tumor tissue was no longer compact as in controls, and numerous fragmented nuclei were present. By 4 days necrosis was almost complete and, at 7 and 15 days, tumors were reduced to partially calcified, necrotic masses with multinucleate giant cells. These changes were confined to regressing tumors and were not seen during tumor growth.

Introduction

Few agents have been as extensively studied in biologic systems as actinomycin D (11, 12). Its importance was 1st recognized when it was shown to inhibit the growth of certain transplantable tumors in rodents (3, 8, 15). Subsequently, the agent achieved great prominence because of its therapeutic effects in Wilms tumor and in choriocarcinoma and because of its capacity to inhibit ribonucleic acid synthesis and thereby block genetic expression. Presumably there is a causal relation between the biochemical action and the useful effect against tumors. Despite this supposition it is not yet clear why the agent should destroy only a select spectrum of experimental and clinical tumors and why even this should occur without the simultaneous and intolerable loss of normal proliferative tissues which are also dependent on nucleic acid synthesis.

We consider of some importance the study of factors responsible for the selectivity of action of actinomycin D against neoplastic tissue in vitro. For this purpose we have been working with the Ridgway osteogenic sarcoma (ROS) in mice which is highly susceptible to destruction by the agent (2, 6). Actinomycin D is also injurious to normal, proliferating tissues (8, 9, 14) and its actions against ROS must, therefore, occur without excessive or irreversible loss of bone marrow, lymphoid organs, and intestinal epithelium. The present report is concerned with the dose-response relationship in well-established ROS tumors which were treated with single injections and were observed for changes in size and histology. The lesions are compared with those found in spleen, thymus, and small intestine with respect to time of onset, duration, and recovery. Evidence has also been sought for the possible assistance by host immune mechanisms in the total obliteration of the tumor. Such findings are essential to an understanding of the differences in biochemical changes induced in tumor and in normal proliferative tissues; these will be the subject of a future communication.

Materials and Methods

Ridgway osteogenic sarcoma was obtained from Dr. Franz A. Schmid of this institute and was carried in female AKR or AKD2F mice from Jackson Memorial Laboratory. Stock tumors were transplanted aseptically every 2 or 3 weeks by s.c. trocar injection of pieces (1–2 mm) into the axillary region of mice weighing 16–18 gm. For 4–5 days after implantation, mice were given drinking water to which was added oxytetracycline (Pfizer) at a concentration of 7.5 gm/liter. Tumors used in experiments were similarly implanted; they grew and were lethal in all untreated animals. Except where noted otherwise, all experiments began during the 3rd week after implantation. Tumor size was estimated in situ with calipers by measurements at the maxima in 2 dimensions; results were averaged and are referred to as average diameter. Mice were killed by cervical dislocation and sections taken for histopathology were fixed in Bouin’s solution, cut at 7 \( \mu \), and stained with hematoxylin and eosin.

Before these experiments began the tumor had been passed 6 times in AKR mice. At this time implants grew rapidly and showed minimal gross necrosis. After the initial experiments of Charts 1 and 2 had been completed, the tumor line began to deteriorate (during summer months when respiratory infections were common among the AKR hosts). Occasional groups of tumors ulcerated in most animals within 2–3 weeks after implantation. Necrosis became extensive and growth rate was erratic.
Results

GROWTH OF ROS AND SURVIVAL AFTER ACTINOMYCIN D. Effects of actinomycin on the growth of ROS tumor are shown in Charts 1 and 2. Animals received a single i.v. injection of actinomycin D on Day 0 (during the 3rd week after implantation), and tumor growth and body weight were recorded at intervals for at least 100 days or until death. All control mice died during the 30-day period following injection. After 100 and 200 μg/kg, tumor growth ceased for about a week and then resumed at a rate comparable to that of control tumors (Chart 1). There were no 100-day survivors among these or control groups and prolongation of survival after doses of 100 and 200 μg/kg did not differ markedly from controls (Chart 2). After 400, 800, and 1200 μg/kg, tumor regression occurred during the 1st 4 weeks. The optimal and most consistent dose was 800 μg/kg. Out of 8 mice that received 400 μg/kg, 5 survived 100 days while 3 died earlier with large, growing tumors. (In other experiments this dose produced fewer or no complete regressions; see below.) Growth curves for tumors in mice that received 1200 μg/kg were the same as for those that received 400 and 800 μg/kg and therefore are not shown in Chart 1.

Chart 2 shows that 800 μg/kg was optimal for 100-day survival: 15 surviving out of 16 treated. In these and all other 100-day survivors there were no palpable tumors at the end of the observation period. Microscopic examination of subcutaneous tissue from surviving mice revealed only calcification at sites formerly occupied by growing ROS. After 1200 μg/kg there was one early death out of 8; 6 survived 100 days. After 1600 μg/kg all 8 mice died within 10 days of treatment; such early deaths are attributed to drug toxicity.

EFFECTS OF I.P. ADMINISTRATION ON TUMOR GROWTH. In another set of experiments actinomycin (800 and 1200 μg/kg) was injected i.p. as before; 80% or more of the treated mice survived and were tumor-free after 100 days (Table 1). If the i.p. route was used instead, the drug was more toxic and there were no 100-day survivors after a dose of 1200 μg/kg. These results indicate that the dose range of therapeutic effectiveness is limited by the route of administration. In the following histopathology experiments, we attempted to probe further into the effect of route of administration on the response of tumor as well as of some tissues that are damaged by actinomycin D.

HISTOLOGIC OBSERVATIONS. Tumor-bearing mice were given actinomycin (800 mg/kg) by i.v. or by i.p. route or were untreated, concomitant controls. Each group consisted of 48 AKR mice bearing tumors from stock grown previously in AKD2F1.
Actinomycin D Effects on Sarcoma in Mice

TABLE 1
LETHALITY AND TUMOR RESPONSE AFTER PARENTERAL ADMINISTRATION OF ACTINOMYCIN D TO AKR MICE

<table>
<thead>
<tr>
<th>Dose and route</th>
<th>Survivors(a)</th>
<th>Day of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1200 µg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p.</td>
<td>0/10</td>
<td>6, 6, 7, 7, 7, 8, 8, 9, 41</td>
</tr>
<tr>
<td>ii.v.</td>
<td>8/10</td>
<td>9, 50b</td>
</tr>
<tr>
<td>800 µg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p.</td>
<td>9/10</td>
<td>69b</td>
</tr>
<tr>
<td>ii.v.</td>
<td>9/10</td>
<td>41b</td>
</tr>
<tr>
<td>400 µg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p.</td>
<td>0/10</td>
<td>20b, 22, 24, 25, 25, 31, 35, 38, 43, 58b</td>
</tr>
<tr>
<td>ii.v.</td>
<td>1/10</td>
<td>17, 21, 21, 22, 24b, 28b, 30b, 35, 39b</td>
</tr>
<tr>
<td>Untreated</td>
<td>0/11</td>
<td>7, 13, 14, 16, 17, 21, 22, 25, 27, 28</td>
</tr>
</tbody>
</table>

\(a\) Of 100 days or more.

b Died with growing tumor after regression (i.e., to less than 0.5 initial average tumor diameter).

hybrids. Eight mice from each group were sacrificed for histopathology at the following times after injection: 24 and 48 hr; 4 days, 7 days, and 15 days. Mice from both treated groups were also killed at 21 days, although no controls were alive at this time. Tumor and small intestine from all mice were examined; in addition, thymus and spleen from at least 4 animals in each group were studied. (Liver, small intestine, and tumor from other experiments were also examined at 4, 24, and 96 hr after similar treatment.) In at least 3 sections of tumor from each mouse, an attempt was made to evaluate the areas of necrosis, the amounts of karyorrhexis, and the general appearance of viable tumor.

Among all controls from 24 hr to 15 days, karyorrhexis was virtually absent in those areas where cells were viable (Figs. 1, 2). However, the areas which were necrotic were estimated to range from 10 to 80% of the total area of sections from these tumors. Necrotic areas were generally irregular in shape but sharply demarcated from viable tissue. In viable areas, cells were round or polygonal with numerous mitotic figures. The pattern formed by these cells was that of a uniformly compact fabric (Fig. 2). In other experiments, the microscopic appearance of untreated tumors grown in AKD2F1 and in AKR mice was the same; similarly 4 hr after i.p. treatment, tumors of both lines had the same appearance as untreated controls.

Twenty-four hr after administration of actinomycin there were changes in tumor morphology. In viable areas fragmented nuclei were prominent; moreover, tumor cells were no longer compact presenting rather the appearance of a loose fabric with separation of cells (Figs. 3, 4). By 48 hr approximately 90% of each tumor was completely necrotic and where necrosis was incomplete the separation of cells was more marked and nuclear fragments were abundant. Those cells that were less affected had hyperchromatic or pyknotic nuclei (Figs. 5, 6). At 4, 7, and 15 days, tumors from treated mice were almost totally necrotic and by 7 days many of the necrotic tumors were partially calcified with multinucleated giant cells (Fig. 7). At 21 days 2 out of 8 mice treated i.p. had growing tumors with microscopic apperances similar to controls: compact viable cells without fragmented nuclei. In contrast the other 6 mice and the 8 mice treated i.v. had tumors which were no longer measurable and which were identifiable only by calcified areas surrounded by a few giant cells (Fig. 8).

Results of microscopic examination of host tissues were generally consistent with previous findings in mice and rats (8, 9, 13, 14). Liver sections appeared the same as those from untreated mice except at 4 and 24 hr after actinomycin when small amounts of karyorrhectic debris were present in the sinusoids (13). There were no changes in thymus until 4 days at which time there were decreased numbers of cortical cells and loss of the cortico-medullary demarcation. At 7 days thymuses were involuted. In small intestine numerous nuclei in the crypt cells were karyorrhectic at 4 and 24 hr. At 24 and 48 hr the remaining nuclei in the crypts were swollen and irregular (9, 14). By 48 hr karyorrhexis was no longer apparent and by 4 days the intestinal epithelium was repaired. In these tissues (liver, thymus, and small intestine) the route by which actinomycin had been administered did not alter the lesions seen or the time of recovery. In spleen, however, the route of administration did affect recovery time. At 24, 48, and 96 hr spleens from all treated mice had decreased numbers of nucleated elements in the red pulp (8, 9). At 7 and 15 days i.v. treated mice had recovered with normal numbers of nucleated elements in the red pulp. In contrast i.p. injected mice at 7 days after actinomycin still had reduced numbers of nucleated cells (with some new foci of hematopoiesis); even at 15 days nucleated cells were fewer than normal.

GROWTH OF TUMORS IMPLANTED AFTER TREATMENT OF MICE. It is important to ask whether regression of the tumor after a single i.v. injection might be due, at least in part, to immunologic mechanisms acting on tumor cells. It seemed possible that antibody reactions might destroy any viable cells remaining after the agent weakened them or slowed their growth. To test for this in animals with regressing tumors, 4 groups of 10 AKR mice each were employed as follows. Groups I and II did not receive tumor implant initially; Groups III and IV were implanted with ROS taken from mice of the AKD2F1 strain. Seventeen days later Groups I and III received actinomycin (800 µg/kg) i.v.; Groups II and IV were injected with saline. The day of injection is referred to as Day 0. Fourteen days thereafter mice in all groups were implanted with challenge tumors (again, from AKD2F1). At this time, mice had recovered from weight loss, presumably from intoxication and from histologic changes in spleen, thymus, and intestine. Experimental conditions and days of death for mice in each group are shown in Table 2; tumor growth curves are shown in Chart 3.

All 8 saline-treated mice of Group IV were dead by day 33 (2 others were sacrificed for histopathology). In this group, 3 lived long enough to demonstrate growth of the challenge tumor, and all died with large, growing tumors at the initial implant site. All actinomycin-treated mice of Group III had regressing tumors when challenged on Day 14; their initial tumors continued to regress while the challenge tumors grew in all mice. The challenge tumor also grew in all mice in Groups I and II. Microscopic appearance of challenge tumors was similar for Groups I, II, and III. Regressing tumors of Group III (at Day 35) were histologically identical to the treated tumors described above. It is known that actinomycin delays antibody induction without
TABLE 2
Experimental Conditions and Lethality of Ridgeway Osteogenic Sarcoma Tumor in AKR Mice (See text for description)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>1st tumor implant</th>
<th>2nd tumor implant</th>
<th>DAY OF DEATH*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
<td>Day 0</td>
<td>Day 14</td>
</tr>
<tr>
<td>I</td>
<td>Actinomycin D</td>
<td>+</td>
<td>42, 43, 47, 49, 54, 56</td>
</tr>
<tr>
<td>II</td>
<td>Saline</td>
<td>+</td>
<td>42, 44, 48, 49, 55, 56</td>
</tr>
<tr>
<td>III</td>
<td>Actinomycin D</td>
<td>+</td>
<td>40, 43, 47, 51, 53, 54</td>
</tr>
<tr>
<td>IV</td>
<td>Saline</td>
<td>+</td>
<td>14, 21, 23, 24, 26, 27, 29, 33</td>
</tr>
</tbody>
</table>

* On Day 35, 4 mice each from Groups I, II, and III were sacrificed for histopathology; on Day 14, 2 mice were sacrificed from Group IV.

Discussion

We have confirmed the inhibition of well-established ROS in mice for prolonged periods (3, 8, 15) and demonstrated the complete destruction of tumor by single, nonlethal doses of actinomycin D. Regression of the tumor is associated with cytologic damage which is apparent within 24 hr and which progresses steadily to almost total necrosis within 4 days. Animals in which treated tumors are regressing accept fresh implants of ROS that subsequently grow well. The rapid development of tumor necrosis and the lack of resistance of the treated host to tumor reimplantation strongly suggest that the chemotherapeutic effect of actinomycin takes place without assistance by immunologic mechanisms.

Host proliferative tissues such as crypt epithelium in intestine, spleen, and thymus are also damaged and partially depleted by doses of actinomycin which destroy ROS tumors. Although intracellular concentrations of the agent do vary, it may be presumed that in each tissue cytotoxicity is ultimately due to the binding of actinomycin to DNA (1, 4, 5). Nevertheless, the defects caused in normal tissue are repaired in time to prevent death of the host. Following i.p. injections splenic injury is more prolonged than after i.v. treatment; presumably this accounts for the greater toxicity of the agent when given by the i.p. route in mice.

The irreversible damage produced in ROS, in contrast with the capacity for recovery in normal proliferative tissues, appears to be the focal point for an understanding of the specificity of action of actinomycin against this tumor. One factor which may be of cardinal importance is the innate instability of ROS tumor cells. This property of ROS may help us to understand the differential action of actinomycin. Histologic inspection of untreated tumors (Fig. 1) reveals the presence of large regions of necrosis and suggests that much of the proliferative activity of the tumor is expended in the production of cells which do not survive. As a consequence the self-maintaining, viable population of tumor cells may be in a continuous state of high proliferative activity. In normal proliferative tissues, however, there is reason to suspect that a significant portion of progenitive cells exist in a state of mitotic dormancy (in the manner of the “no cell cycle” liver cells and the “available stock” of bone marrow stem cells of Lajtha et al. (7) or the G0 cells of the intestinal mucosa of Quastler (10)). These dormant members of a cell line provide a suitable reserve which can be triggered into proliferation when there is need to repair a deficiency in the tissue caused by destruction of cells by exogenous toxic agents. In the present context the importance of a mitotically dormant reserve may lie in the fact that nonproliferating cells are less susceptible to damage by actinomycin (9, 13, 14). Thus, a significant number of dormant progenitive cells may survive and repopulate normal proliferative tissues after doses of actinomycin which are uniformly lethal to all or most ROS cells.

Another aspect of the susceptibility of ROS that may be related to its innate instability has been seen in studies of its nucleic acid metabolism after actinomycin treatment. A loss of ribonucleic acid takes place which is rapid by comparison with that occurring in other tissues such as liver and small intestine. The loss occurs even though incorporation of precursors into ribonucleic acid is not completely blocked and without concurrent change in tumor DNA content (unpublished observations). If the rate of turnover of RNA in ROS cells is unusually high by comparison with that of less responsive tumors or of normal tissues, this loss could be involved in the high susceptibility.
References


Stock tumor from AKD2Fi mice was implanted in AKR mice 17 days before the experiment. At that time mice were killed (Figs. 1, 2) or treated with actinomycin D and then killed when indicated (Figs. 3–8).

Fig. 1. Section of untreated tumor at 17 days after implantation. The irregular areas of necrosis are well defined and delineated from the intact and viable portions of tumor. × 50.

Fig. 2. Viable area of untreated tumor 17 days after implantation. Note generally uniform appearance and distribution of cells, many mitotic figures, and absence of necrosis. × 230.

Fig. 3. Tumor 24 hr after treatment with actinomycin D. Areas of complete necrosis at top right and side as in control. However, in the generally viable areas, there is looseness and separation of the tumor cells. Compare with Fig. 1. × 50.

Fig. 4. Tumor 24 hr after treatment with actinomycin D. In areas not completely necrotic, the tumor cells are loosely arranged and separated. Note intermixture of karyorrhectic material. Compare with Fig. 2. × 200.

Fig. 5. Tumor 48 hr after treatment with actinomycin D. Nearly all tumor cells are necrotic. × 50.

Fig. 6. Tumor 48 hr after treatment with actinomycin D. In this particular tumor about 90% was necrotic and the area selected was from portion in which tumor cells were still recognizable. The nuclei of the tumor cells are hyperchromatic and are surrounded by much karyorrhectic debris. × 230.

Fig. 7. Tumor 15 days after treatment with actinomycin D. There are multinucleated giant cells around necrotic material. × 230.

Fig. 8. Tumor 21 days after treatment with actinomycin D. Multinucleated giant cells are intermixed with calcified material in subcutaneous tissue. Dark linear structures at top are hair follicles at skin surface. × 160.
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