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Regional chemotherapy in an experimental model of Wilms' tumor in rats

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Summary. A s.c. experimental model of Wilms' tumor in rats was used to compare the effects of intratumoral treatment with vincristine plus actinomycin D to i.v. treatment with these chemotherapeutic drugs. The Wilms' tumor model is a fast-growing solid tumor that has been shown to be resistant to traditional clinical treatment procedures used for Wilms' tumor in man. Injection of the chemotherapeutic drugs directly into the tumor mass was found to be more effective than i.v. therapy in causing long-term remission of the tumor. Intratumoral therapy was also less toxic to the animals than i.v. therapy when measured by post-treatment survival rates and weight loss during the 1st week following treatment. However, intratumoral treatment caused an initial fibrosis of the tumor tissue, which resulted in a slower rate of absorption of the resultant fibrotic tumor mass than was seen in tumors treated i.v. Also, intratumoral injection resulted in necrosis of the overlying skin, which healed as the fibrotic tumor tissue was absorbed. Intratumoral treatment of a cervical tumor was found to cause the remission of a second major tumor mass located at some distance from the initial injection (i.e., in the lumbar region). No significant benefits were noted when dimethylsulfoxide (DMSO) was used in place of aqueous mannitol as a vehicle to deliver the chemotherapeutic agents. There was a significant correlation between the drug dose-to-tumor-size ratio (D/T ratio) and the effectiveness of the chemotherapy. When this ratio was high enough, a single treatment with a combination of vincristine and actinomycin D usually resulted in total remission of the experimental Wilms' tumor in response to either intratumoral or i.v. therapy.

Introduction

Unlike whole-body chemotherapy, regional cancer chemotherapy implies that the chemotherapeutic agent is applied to a small region of the body wherein a cancer resides. The benefits of regional therapy are that it concentrates chemotherapeutic agents in regions intended for treatment, it can overcome barriers to the distribution of a drug, and it may lessen the deleterious side effects that otherwise result from the dissemination of such agents throughout the body. Regional chemotherapy has been successfully applied to a number of conditions including intracavity therapy of bladder cancer [8], intrathecal treatment of brain tumors [5], topical treatment of skin lesions [16], and a variety of regional vascular perfusion procedures wherein the immediate blood supply to the cancerous region is focally perfused [14]. Regional chemotherapy involving the injection of chemotherapeutic agents directly into solid tumors has been applied to a lesser extent and with variable success. For example, Bateman has reported the successful treatment of solid tumors including breast cancers by intratumoral injection of thio-TEPA [1]. Other investigators, however, have reported that intratumoral injection of chemotherapeutic agents into human cerebral tumors has not been effective [14]. Among the proposed disadvantages of regional chemotherapy are that such treatment may not effectively dissipate chemotherapeutic agents to tumor cells that are at some distance from the site of focal treatment, and the potential for focal necrosis in response to high local concentrations of these agents.

In the present investigation, we compared the effectiveness of intratumoral with i.v. chemotherapy in a s.c. model of Wilms' tumor, the effects of regional injection in one tumor site with its effects on a second noninjected tumor site, and the effects of aqueous mannitol solutions vs dimethylsulfoxide (DMSO) as intratumoral injection vehicles. Wilms' tumor, also known as nephroblastoma, is a tumor mainly found in children, representing 6% of the malignant neoplasms seen before the age of 15 years. The Wilms' tumor model that we used has been extensively characterized, and its response to surgical excision, radiation treatment, chemotherapy, and a combination of these treatments have previously been reported [6, 9, 10, 15]. These studies have indicated that this tumor model is in most ways similar to Wilms' tumor in humans, except that it appears to be more resistant to therapy [6, 9, 11]. For example, it has been shown that postoperative chemotherapy following surgical removal of the tumor failed to prevent recurrence of the primary tumor or improve survival rates [6]. In view of the resistance of this tumor model to traditional treatment procedures for Wilms' tumor, it has been suggested that this tumor may represent a good experimental model in which to evaluate treatment procedures that might be used in treating resistant Wilms' tumor in man [6, 9]. In the present study, we found that single-dose intratumoral chemotherapy with a combination of actino-

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mycin D and vincristine was more effective than whole-body i.v. treatment with these same compounds in treating this experimental model of Wilms' tumor. We also found that with smaller tumors, a single i.v. or intratumoral treatment with a high-dose combination of actinomycin D and vincristine can produce total remission of the tumor.

Materials and methods

Inbred male Wistar-Furth rats weighing between 200 and 250 g were used in these investigations. The experimental Wilms' tumor was kindly provided by Dr. Michael McGarry of the Roswell Park Memorial Institute. All experiments in this study were carried out by s.c. transplantation of tumor tissue approximately 2×4 mm in size into the cervical and lumbar regions on the dorsal aspect of the rats. Most of the rats received aqueous solutions containing mannitol (20 mg/cm^3), in which was dissolved equal amounts by weight of vincristine (Sigma) and actinomycin D (Sigma). Both intratumoral and i.v. injections of the chemotherapeutic agents were given under ether anesthesia using a 1-cc tuberculin syringe fitted with a 25-gauge needle. Intratumoral injections were delivered into the central core of the tumor, whereas i.v. injections were given via the femoral vein. Groups of tumor- and non-tumor-bearing rats received either 100 or 400 μg/kg of the chemotherapeutic agents. In rats receiving intratumoral therapy, the injections were always given into the cervical tumors, leaving the lumbar tumors noninjected. Rats receiving the higher dose of chemotherapy (i.e., 400 μg/kg) were further subdivided into those with high D/T ratios and those with low D/T ratios. That is, heavier rats with smaller tumors received more chemotherapeutic agent per unit tumor size (i.e., had high D/T ratios) than smaller rats with larger tumors. Finally, in another group of rats we evaluated the effects of i.v. or intratumoral injection of the chemotherapeutic agents (400 μg/kg) dissolved in DMSO. All rats received care in accordance with institutional and NIH guidelines.

The data are reported as the mean ± 1 standard error (SEM). Differences between groups were compared using Student's *t*-test. A *P* value of ≤ 0.05 was considered statistically significant.

In some cases, histological evaluation of the tumors was undertaken to help determine the composition of tumor masses that appeared to be firm and fibrotic upon palpation. For these studies, small samples of tissue (2×2 mm) were excised and immediately immersed in 2% phosphate-buffered glutaraldehyde for several hours. The tissue was rinsed in phosphate buffer, postfixed in 1% Os O_4 for 45 min, dehydrated through graded acetones, and embedded in Spurr embedding medium. Thick sections ($1-2$ microns) were taken, stained with toluidine blue, and examined under an Olympus BH-2 light microscope.

Results

The s.c. Wilms' tumor in rats not treated by chemotherapy exhibited different growth rates depending on their sizes. When the initial tumor was approximately 0.5 cm^3 in size, it exhibited a mean increase in size of approximately 4,000% in the period of 1 week. However, once the tumors reached the size of aproximately 15 cm^3, they increased in size by approximately 200% in 1 week. Tumor growth was therefore initially very rapid but slowed as the tumor mass became larger and the central core of the tumor became necrotic. Although both cervical and lumbar tumors were implanted at the same time using the same size of tumor, lumbar tumors usually developed slower than cervical tumors, such that cervical tumors were often ten or more times larger than lumbar tumors at the time of treatment. Nontreated control rats with double tumors (i.e., cervical and lumbar tumors) similar in size to those studied in most of the following experiments usually died within 2 weeks (Table 1). Although gross examination did not reveal metastasis to the kidneys, liver, spleen, or lungs, a careful histologic examination of these organs was not undertaken and micrometastasis to these regions cannot be excluded.

The effects of the various treatment protocols on survival rates, body weights, and remission of the cervical and lumbar tumors are summarized in Tables 1–5. The i.v. treatment of non-tumor-bearing rats with low concentrations of vincristine and actinomycin D (i.e., 100 μg/kg) resulted in a loss of weight during the first several days following treatment, followed by the gradual regaining of this lost weight within 1 week posttreatment. The i.v. treatment with 400 μg/kg resulted in the death of two of the

Table 1. Percentage survival following different treatment procedures with vincristine plus actinomycin D

Experimental group	Rats (*n*)	1st week	2nd week	3rd week	5th week	Long term >50 days
Tumors No therapy	5	100%	0	0	0	0
No tumors 100 μg/kg i.v.	5	100%	100%	100%	100%	100%
No tumors 400 μg/kg i.v.	5	60%	60%	60%	60%	60%
Tumors 100 μg/kg i.v.	5	100%	100%	0	0	0
Tumors 400 μg/kg i.v. low D/T ratio	9	89%	89%	89%	22%	0
Tumors 400 μg/kg i.v. low D/T ratio DMSO	5	100%	60%	40%	0	0
Tumors 400 μg/kg i.v. high D/T ratio	10	60%	50%	50%	50%	50%
Tumors 100 μg/kg intratumorally	5	100%	80%	0	0	0
Tumors 400 μg/kg intratumorally low D/T ratio	9	100%	89%	78%	33%	22%
Tumors 400 μg/kg intratumorally low D/T ratio DMSO	4	100%	75%	75%	25%	25%
Tumors 400 μg/kg intratumorally high D/T ratio	10	100%	100%	100%	50%	50%

D/T ratio: ratio of the dose of chemotherapeutic agent (in μg) to the size of the cervical tumor (in cm^3)

Table 2. Percentage change in body weights during the first 8 days following different treatment procedures with vincristine and actinomycin D

Experimental group	Day 2 (SEM)	Day 4 (SEM)	Day 6 (SEM)	Day 8 (SEM)
No tumor No therapy	1.2% (0.5)	8.4% (0.6)	11.2% (1.2)	12.4% (1.2)
No tumors 100 μg/kg i.v.	−2.0% (0.6)	1.8% (0.8)	2.6% (0.7)	7.2% (1.0)
No tumors 400 μg/kg i.v.	−9.4% (0.5)	−18.0% (0.6)	−10.0% (3.0)	−8.7% (1.2)
Tumors 100 μg/kg i.v.	−0.2% (0.6)	7.4% (0.9)	12.2% (1.2)	19.0% (1.7)
Tumors 400 μg/kg i.v.	−8.6% (1.4)	−10.0% (1.5)	−3.8% (1.4)	−2.6% (1.1)
Tumors 100 μg/kg intratumoral	4.2% (1.7)	12.2% (2.5)	16.6% (2.4)	22.6% (3.2)
Tumors 400 μg/kg intratumoral	−5.8% (0.5)	−4.6% (2.5)	1.6% (0.8)	0.6% (4.9)

SEM, standard error of the mean

five animals within 2 days and a significant loss of weight and diarrhea in the remaining rats. It took approximately 2 weeks for the surviving rats to reach their original weights.

The i.v. treatment of tumor-bearing rats at low doses of vincristine and actinomycin D (i.e., 100 μg/kg) did not result in a significant remission in the sizes of the growing tumors. Intratumoral treatment with low doses of vincristine and actinomycin D appeared to result in some remission in the growth of the injected tumors; however, the extent of this remission was not statistically significant. Also, the noninjected lumbar tumors in these rats grew rapidly, and all of the rats in this group died before the end of the 3rd week posttreatment.

In rats treated with 400 μg/kg, survival and the extent of remission of tumor growth correlated with both the method of drug administration (i.e., i.v. intratumoral) and the D/T ratios. That is, large rats with small tumors received considerably more chemotherapeutic agent per unit tumor mass (i.e., had higher D/T ratios) than smaller rats with larger tumors. In ten rats that received i.v. treatments

Table 4. Percentage change in the size of lumbar tumors following intratumoral treatment of cervical tumors or i.v. injection of vincristine and actinomycin D (400 μg/kg body weight)

Experimental group	1st week (SEM)	2nd week (SEM)	3rd week (SEM)
i.v. Low D/T ratio	20.3% (13.9)	652.8% (200.0)	2015.0% (310.7)
i.v. High D/T ratio	−36.0% (22.4)	−95.0% (2.3)	−100.0% (0)
Intratumoral Low D/T ratio	38.0% (47.0)	398.0% (178.9)	1191.4% (481.5)
Intratumoral High D/T ratio	−24.5% (18.6)	274.1% (225.1)	1072.2% (482.5)

D/T ratio: ratio of the dose of chemotherapeutic agent (in μg) to the size of the cervical tumor (in cm^3). SEM, standard error of the means

Table 3. Mean percentage change in the size of cervical tumors following different treatment procedures with vincristine and actinomycin D

Experimental group	No. Rats	Mean tumor size (cm^3) (SEM)	Mean D/T ratio (SEM)	1st week (SEM)	2nd week (SEM)	3rd week (SEM)	5th week (SEM)	Long term >50 days (SEM)
Tumor Controls	5	10.9 (2.4)		407% (174%)	D	D	D	D
100 μg/kg i.v.	5	12.4 (4.2)	2.6 (0.7)	380% (155)	2478% (1263)	D	D	D
400 μg/kg i.v.	9	17.3 (2.3)	6.5 (1.0)	−54% (6)	5% (37)	153% (121)	1988% (234)	D
400 μg/kg i.v. DMSO	5	12.1 (1.3)	6.8 (0.8)	−37% (39)	405% (116)	5954% (894)	D	D
400 μg/kg i.v.	10	3.0 (0.7)	57.1 (17.1)	−75% (14)	−92% (5)	−94% (9)	−100% (0)	−100% (0)
100 μg/kg intratumorally	5	14.9 (2.4)	1.7 (0.5)	227% (45)	704% (95)	D	D	D
400 μg/kg intratumorally	9	14.5 (2.2)	7.2 (1.1)	−15% (12)	−84% (16) 73% (38)	−44% (35) 246% (47)	−100%F (0)	−100%F (0)
400 μg/kg intratumorally; DMSO	4	19.1 (3.7)	4.8 (1.3)	26% (68)	−17% (16)	25%F (15)	−100%F (0)	−100%F (0)
400 μg/kg intratumorally	10	4.8 (0.8)	41.8 (14)	47% (38)	166%F (113) 76% (66)	132%F (83) 715% (13)	−100%F (0)	−100%F (0)

D/T ratio: ratio of the dose of chemotherapeutic agents (in μg) to the size of the cervical tumors (in cm^3). SEM, standard error of the mean; F, the tumors in this subgroup exhibited extensive fibrosis; D, all rats died

Table 5. Percentage of tumors exhibiting long-term remission (>50 days) following different treatment procedures with vincristine plus actinomycin D

Experimental group	D/T ratios (SEM)	Cervical tumor	Lumbar tumor
100 μg/kg i.v.	2.6 (0.7)	0	0
400 μg/kg i.v.	6.5 (1.0)	0	0
400 μg/kg i.v. DMSO	6.8 (0.8)	0	0
400 μg/kg i.v.	57.1 (17.1)	50%	50%
100 μg/kg intratumorally	1.7 (0.5)	0	0
400 μg/kg intratumorally	7.2 (1.1)	50%	22%
400 μg/kg intratumorally DMSO	4.8 (1.3)	50%	25%
400 μg/kg intratumorally	41.8 (13.8)	80%	50%

D/T ratio: ratio of the dose of chemotherapeutic agent (in μg) to the size of the cervical tumor (in cm^3). SEM, standard error of the means

with mean D/T ratios of 57 (±17 SEM), 50% of the rats exhibited apparently complete loss of their tumors and were long-term survivors. The remaining rats died during the 1st week posttreatment, apparently due to excessive drug toxicity. Similarly, 50% of the rats receiving intratumoral treatment with mean D/T ratios of 42 (±14 SEM) were also long-term survivors, exhibiting apparently full remission of both injected cervical and noninjected lumbar tumors. In addition, three of the remaining five animals exhibited full remission of the injected cervical tumor, but apparently the continued growth of the noninjected lumbar tumors resulted in their death. The remaining two rats exhibited reduced growth of the injected cervical tumors, but died due to significant growth of the noninjected lumbar tumors. Unlike rats treated i.v. with 400 μg/kg, none of the rats in the intratumorally injected group died of apparent drug toxicity and all survived for more than 3 weeks following chemotherapy. It is important to note that intratumorally treated tumors first appeared to grow in the 1st week posttreatment and then shrank at a much slower rate than the tumors of rats treated i.v. (Table 3). Histologic examination revealed an extensive fibrosis in intratumorally treated tumors, which as a result took considerably longer to be absorbed. Also, it should be noted that intratumoral treatment resulted in necrosis of the overlying skin: a scab formed on the skin at the site of the intratumoral injection and eventually sloughed as the skin healed.

In rats receiving 400 μg/kg i.v. treatment at lower D/T ratios, there was significant initial remission in tumor growth, but all the tumors eventually started to grow again and none of the rats survived for as long as 5 weeks posttreatment. Rats receiving 400 μg/kg intratumoral treatment at low D/T ratios fared better, with four of nine rats exhibiting fibrosis and apparently total remission of the injected tumors. However, three of the four in this latter group eventually died, apparently due to rapid growth of the noninjected lumbar tumors. The fourth rat exhibited apparently total remission of both cervical and lumbar tumors and was a long-term survivor.

The i.v. injection of the rats with vincristine and actinomycin D dissolved in DMSO did not have a significant effect on the outcome of the treatment. In these experiments, the D/T ratios were small and both groups behaved similarly to rats treated with the chemotherapeutic agents dissolved in mannitol solutions.

Discussion

In this investigation we evaluated the effectiveness of intratumoral treatment with a combination of vincristine and actinomycin D in a s.c. model of Wilms' tumor. To our knowledge, neither vincristine nor actinomycin D has previously been used in a intratumoral treatment regime. Our findings indicate that this method of chemotherapy appeared more effective than i.v. treatment in inducing remission of this fast-growing experimental tumor.

It is perhaps not surprising that intratumoral treatment as applied in the present study was successful. According to the classic work of Skipper et al. [13], a given dose of chemotherapeutic agent follows first-order kinetics and kills a constant fraction of cells, regardless of the number present. In a large, rapidly growing tumor such as that evaluated in the present study, it is therefore necessary to expose the tumor to a large dose of chemotherapeutic agents to kill a high percentage of the tumor cells. If even a small percentage of cells making up such a large tumor mass is permitted to survive, it would represent a considerable number of cells that could more easily overcome protective host factors (e.g., immunologic mechanisms) and reestablish the tumor. Injection of these chemotherapeutic agents directly into the tumor results in higher concentrations of these agents being trapped in the parenchyma of the tumor for a longer time than with i.v. therapy. Also, intratumoral injection has the potential of delivering these agents to the necrotic centers of such tumors, which might otherwise represent "tumor sanctuaries" difficult to reach by i.v. therapy. Finally, one of the objections to regional therapy has been that it might not effectively treat cancers that have spread to more than one site. In the present study, we found that a second noninjected tumor site was significantly affected by regional treatment. However, it should be noted that in our studies both the treated and nontreated tumors were located s.c., and it is not clear from these studies how effective intratumoral treatment of s.c. tumors might be in causing remission of secondary tumors located in more distal regions of the body (e.g., lungs, liver). No additional benefit was found in using DMSO as an injection vehicle, although it has been proposed that it might improve the effectiveness of chemotherapeutic agents such as vincristine and be an effective vehicle for delivering such agents to cells [12].

The success of the intratumorally treated tumor in the present investigation argues for attempting to use such a technique in those cases in which it is otherwise risky to remove a tumor mass by surgical incision, or perhaps prior to surgical excision in an attempt to reduce the active tumor mass and/or metastasis due to tumor rupture and tumor cell spillage. That intratumoral injection of chemotherapeutic agents can be applied clinically with success has previously been shown by Bateman [1], who reported

clinical cases in which intratumoral injection of thio-TEPA appeared to induce complete remission of breast cancer.

Previous investigations studying the experimental model of Wilms' tumor used in the present investigation have indicated that multiple low doses of a combination of vincristine and actinomycin D are not effective in treating this experimental tumor, even following surgical excision of the tumor [6]. Also, previous attempts to treat this experimental tumor with multiple or single high doses of vincristine or actinomycin D injected separately have had limited success in inducing full remission of the tumor without resulting in high mortality [11]. Our observations using i.v. treatment with low-dose combination therapy using actinomycin D and vincristine confirm the inadequacy of this treatment regime. However, our study indicates that long-term remission of this fast-growing tumor can be achieved with a single administration of relatively high concentrations of a vincristine-actinomycin D combination when the D/T ratio is high (i.e., when the tumor is small relative to the delivered dose). Intratumoral injections in small cervical tumors resulted in full remission of 80% of these tumors while reducing the toxic side effects that otherwise result from i.v. treatment. Had these rats not had large lumbar tumors, or had the lumbar tumors also been treated, it is likely that survival in the intratumorally treated groups would have been higher. The favorable response of the tumors to a combination of vincristine and actinomycin D as opposed to the separate use of these drugs [11] is in line with national Wilms' tumor studies [3, 4], which have reported that these drugs are significantly more effective when used in combination than when used separately. Also, the effectiveness of the vincristine-actinomycin D combination in the Wilms' tumor model is another indication that this experimental model is in many ways similar to Wilms' tumor in man, representing a valuable model to use in the study of this clinically important tumor.

Our observation that intratumoral chemotherapy significantly reduces the whole-body effects of these chemotherapeutic agents compared with their i.v. injection is in line with other studies indicating that regional therapy has the potential for significantly reducing the whole-body effects of chemotherapy [2, 14]. This observation is of added significance in Wilms' tumor, in that other drugs (i.e., doxorubicin) have been reported to be especially effective against this particular tumor but have toxic side effects that limit their possible use [10]. Future testing the effectiveness of chemotherapeutic agents such as doxorubicin with the regional method of administration would be of value.

Although the present experiments indicate that intratumoral therapy can be effective, there is probably room for improving this procedure, including determining the most effective kind, dose, and combination of drugs to use; exploring the potential of using injection vehicles which might serve to retain the chemotherapeutic agent in the tumor and even further lessen the whole-body effects of intratumoral therapy; adjusting the osmolarity and pH of the injection vehicle to optimize the effectiveness of the chemotherapeutic drugs; determining the optimal volume of solution to inject into a given tumor as well as the proper number of intratumoral injections and timing of these injections if multiple injections are given; and the possibility of using special injector needles [7] and other ancillary procedures (e.g., locally applied heat). The focal necrosis and potential fibrotic masses that result from regional treatment are issues that also should be considered. For example, because extensive fibrosis may form in response to intratumoral treatment (as exemplified in the present study), the tumor mass may not shrink and, indeed, may appear to grow, indicating that care should be taken not to interpret the latter as an indication that the treatment has been ineffective.

In summary, the present study indicates the beneficial effects of intratumoral administration of a combination of vincristine and actinomycin D in treating a resistant experimental model of Wilms' tumor. This method of treatment has potential benefits and may represent the best procedure for treating a variety of tumors that are resistant to other therapeutic procedures. The need remains to delineate the effective amounts and kinds of chemotherapeutic agents to be used in this type of therapy, possibly to improve the injection vehicle and ancillary procedures, and to test the usefulness of this procedure in other tumor models. Nevertheless, the results of the present study are encouraging, underlining the potential usefulness of this procedure.

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