Warfarin Therapy as an Adjunct to the Surgical Treatment of Malignant Tumors in Mice

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SUMMARY

Warfarin anticoagulation throughout the pre-, intra-, and early postoperative periods significantly improved the long-term survival and the cure rate following amputation of a well-established primary tumor. In the case of the tumor system with the more virulent metastatic spread, the T241 sarcoma in C57BL/6N mice, the increase in the cure rate was seven-fold or 8% to 55%. In the C3H/HeN mice with mammary adenocarcinoma, the increase in the cure rate was from 29% to 53%. The weight of the primary tumor-bearing limb was also significantly reduced in the Warfarin-treated mice. The level of anticoagulation producing this improvement was not excessive, with the prothrombin time prolonged between 2.8 and 3.5 times the normal, average value. Deaths from complications were increased in the Warfarin-treated mice, but not nearly to the extent that deaths with metastatic disease were reduced.

INTRODUCTION

Surgical attempts to achieve a cure from a malignancy by ablation of the primary tumor and its local and regional extension are frequently thwarted by metastatic spread to distant areas. Thus, control of metastatic dissemination has been sought as a logical method of improving the cure rate with standard surgical procedures. In a recent study of metastatic spread, long-term oral Warfarin therapy was shown to reduce significantly the incidence of spontaneous pulmonary metastases in two different mouse tumor systems (16). The present study, using the same mouse tumor systems, is an assessment of Warfarin (As Coumadin, Endo Laboratories) anticoagulation as an adjunct to surgical therapy.

MATERIALS AND METHODS

The host-tumor systems used were the Lewis T241 sarcoma in C57BL/6N mice and the mammary adenocarcinoma in C3H/HeN mice. The former was induced in this strain by methylcholanthrene in 1938 and carried by transplantation. The latter, a spontaneous tumor, was in the 123rd transplant generation. Both have a uniform, rapid growth rate, and a metastatic pattern well established by previous study. Metastatic spread in the period studied was confined to the lungs (5). All mice were 6- to 8-week-old inbred females; they were individually housed and were fed Purina laboratory chow. The mice were observed for two weeks to detect any recurrent illness.

Tumors were inoculated subcutaneously into the left thigh. The inoculum was 0.05 ml of a tumor cell suspension prepared by passage of 1.0 gm of grossly viable tumor and 10 ml of 0.85% sodium chloride through a Snell cytosieve. In both strains, 170 mice were inoculated and divided into two equal groups.

Warfarin treatment was started two days later in one group of each strain; it was added to the individual water bottles in a dosage of 9.325 mg/liter of water for the C57 mice and 9.215 mg/liter of water for the C3H mice. These dosages were found to prolong the prothrombin time during a 72-hr period to between two and three and a half times the average value for normal mice, and to maintain this level throughout the duration of therapy (16). Water was taken ad libitum, and Warfarin and water were changed alternately each third and fourth day. Warfarin was continued throughout the period of primary tumor growth and through the second day following amputation.

Amputations were performed on the C57 mice on the 9th day following inoculation and on the 11th day in the C3H mice, i.e., 7 and 9 days respectively after the initiation of Warfarin therapy. These times allowed maximal enlargement of the tumors while still permitting their complete removal by the high thigh amputation. Amputation was performed by cross-clamping the limb close to the trunk, amputating with cutting cautery, and securely approximating the skin edges with metallic clips. All mice were returned to their individual cages and resumed eating and drinking the same day. The tumor-bearing extremities were weighed and examined histologically.

Prothrombin time determinations were done by Miale's micromethod (7) using periorbital blood. Determinations were done on ten mice from each of the four groups on the day of amputation and on five mice from each group on both the day Warfarin was discontinued and the day following. These twenty mice from each group were discarded, as were five additional Warfarin-treated C3H mice who showed middle-ear infection prior to amputation. Thus, 65 mice in both C57 groups and 65 and 60 mice in the C3H groups underwent
amputation and were observed until death or the day of sacrifice.

The mice were observed daily for a period extending to the 60th day after inoculation for the C57 mice and to the 65th day for the C3H strain. Previous study had shown that virtually all primary tumor-bearing mice and those with only metastatic tumor, the primary tumor having been amputated, will die during these intervals (5). All surviving mice were killed on these days. Mice found dead during the follow-up interval and those killed were examined macroscopically for recurrence of a tumor mass at the amputation site, and for pulmonary metastatic spread by India-ink differential staining of normal lung and tumor tissue, as previously described (19).

RESULTS

The Warfarin-treated mice were anticoagulated to a prothrombin time level of 2.8 to 3.5 times the normal average in the pre-, intra-, and early postoperative periods, a total of 10 days of Warfarin therapy for the C57 mice and 12 days for C3H mice. At amputation, the inoculated thighs in all 255 mice were enlarged to between two and three times the normal diameter. The average weight of the amputated tumor-bearing limbs of the Warfarin-treated mice was significantly less in both systems than the weight of the amputated limbs of control mice; the weights were 0.8 gm compared to 2.2 gm in the C57 mouse and 1.1 gm compared to 2.2 gm in the C3H mouse. There was a corresponding difference in gross size of the tumor-bearing thigh, and upon histologic examination there was less tumor tissue and a smaller tumor mass in the thigh of the Warfarin-treated mice.

Mortality data for those mice dying during the follow-up interval are given in Tables 2 and 4. Mortality was subdivided into operative deaths, deaths with recurrence of tumor at the amputation site, and deaths during the follow-up interval with or without evidence of metastatic disease.

Operative deaths were those occurring within 10 days of amputation and in nearly all instances were associated with bleeding from the amputation site. These were increased among the Warfarin-treated mice in both systems, especially in the C3H mice. There were no significant mortality differences between the groups with regard to recurrence of tumor mass at the amputation site or deaths without evidence of metastatic disease.

In the present study, Warfarin therapy was an effective adjunct to amputative surgery. The mechanism by which Warfarin influences primary tumor growth and metastatic tumor spread is not clear. Attention has been called to the importance of the coagulation mechanism, in particular fibrin deposition, in both the growth of a primary tumor and its metastatic spread. Direct cellular effects of Warfarin therapy have also been reported.

Fibrin deposition has been noted in a wide variety of tumors, most prominent at the invading periphery, and is felt to be an important lattice for tumor growth (10). Fibrin has also been detected in many tumors, transplanted and spontaneous, by fluorescent antibody technics (4). Human cancer cells contain an agent “cancer coagulative factor,” which induces fibrin formation and which is now felt to resemble known thromboplastins (9, 17). The action of this coagulative factor from cancer cells can be blocked in humans and rabbits by Warfarin-type anticoagulants (17). In a system similar to the present study (an early transplant generation methylcholanthrene-induced fibrosarcoma in C57BL/6N mice), reduced tumor weight was noted with Warfarin treatment (11). Warfarin therapy produced a marked reduction in size and weight of autochthonous tumors in C57 mice (15). Heparin anticoagulation appeared to have little effect on primary tumor size. Wood et al. (21) recorded a slight reduction in the

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Alive at 60 days</th>
<th>Alive and free of metastases (cured)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amputated only</td>
<td>65</td>
<td>6 (9%)</td>
<td>5 (7%)</td>
</tr>
<tr>
<td>Pre-, intra-, and postamputation anticoagulants</td>
<td>65</td>
<td>38 (59%)</td>
<td>36 (55%)</td>
</tr>
</tbody>
</table>

Combined coumadin-amputation treatment of Lewis T241 in C57BL/6N mice.

*Difference significance of *P* < 0.005, *X²* test.

DISCUSSION

In both of the host-tumor systems, the Warfarin-treated group had significantly increased long-term survival. Tables 1 and 3 show that this increased from 9% to 59% in the C57 system, a six-fold increase (*P* < 0.005, *X²* test), and from 35% to 58% in the C3H strain (*P* < 0.025, *X²* test). The mice found to be free of metastatic disease when killed at the end of the followup period were considered cured of their tumors. Not all long-term survivors were free of metastatic disease, though only a few mice in each of the four groups evidenced metastases when killed, and the groups did not differ significantly in this regard. In the C57-T241 sarcoma system, a cure rate of 8% with amputation alone was increased to 55% (a seven-fold increase) by the addition of Warfarin therapy (*P* < 0.005, *X²* test). In the C3H mice with mammary adenocarcinoma a cure rate of 29% with amputation alone was increased to 53% with Warfarin (*P* < 0.05, *X²* test). The cure rate was significantly improved in both systems by coupling Warfarin treatment with amputation.
Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Operative deaths</th>
<th>Recurrence of tumor</th>
<th>Died free of metastases</th>
<th>Died with metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amputated only</td>
<td>1 (2%)</td>
<td>1 (2%)</td>
<td>2 (3%)</td>
<td>55 (85%)</td>
</tr>
<tr>
<td>Pre-, intra-, and postamputation anticoagulants</td>
<td>3 (5%)</td>
<td>3 (5%)</td>
<td>7 (11%)</td>
<td>14 (22%)</td>
</tr>
</tbody>
</table>

Mortality of combined coumadin-amputation treatment of Lewis T241 in C57B1/6N mice.

*Difference significance of $P < 0.005$, $X^2$ test.

Table 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of mice</th>
<th>Alive at 65 days</th>
<th>Alive and free of metastases (cured)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amputated only</td>
<td>65</td>
<td>23 (35%)</td>
<td>19 (29%)</td>
</tr>
<tr>
<td>Pre-, intra-, and postamputation anticoagulants</td>
<td>60</td>
<td>35 (58%)</td>
<td>32 (53%)</td>
</tr>
</tbody>
</table>

Combined coumadin-amputation treatment of mammary adenocarcinoma in C3H/HeN mice.

*Difference significance of $P < 0.025$, $X^2$ test.

The weight of subcutaneous C150 tumors in mice, and Retik et al. (12) found no inhibitory effect of long-term heparin anticoagulation on primary tumor growth of either T241 or DBA49 sarcomas in mice.

The impairment of primary tumor growth with Warfarin may be a direct cellular effect not mediated through anticoagulant action with reduced fibrin formation (18). Direct effects of Warfarin on cellular metabolism and activity have been reported. Dicoumarol and Warfarin do uncouple oxidative phosphorylation (6). Warfarin selectively inhibits in vivo locomotion of V2 carcinoma cells and has cytotoxic action of murine L1210 leukemia cells in vitro (18).

The influence of Warfarin therapy on the primary tumor may account for the reduced incidence of metastatic spread. However, the belief that Warfarin therapy does indeed modify either the rate of tumor cell embolization or the nature of such emboli is not established. Warfarin's anticoagulant action may be of importance in retarding metastatic spread. Anticoagulation with various drugs reduces the number of embolized tumor cells as well as other embolized particles which lodge in pulmonary capillaries (21). Anticoagulation or fibrinolysis with numerous agents significantly reduced the incidence of metastatic growths following intravenous tumor cell injection, while agents increasing the coagulability of blood increased this incidence (1-3).

Though not identical, it seems possible that this action of anticoagulation would apply to tumor cells spontaneously embolized from a primary tumor and would impair their formation of metastatic growths. The revealing work of Wood et al. (21) on the pathogenesis of metastasis formation assigns a decisive role to the formation of an intracapillary microthrombus about the embolic tumor cells shortly after their initial endothelial adherence. When this enveloping microthrombus failed to form, the cells did not penetrate the endothelium but were dislodged. The fate of such dislodged cells in the general circulation is not known. With anticoagulation, the formation of such microthrombi could be impaired. The relative importance of the direct cellular effects and the indirect anticoagulant action of Warfarin on both primary tumor growth and metastatic spread remains to be clarified.

In view of the possible mechanisms by which Warfarin reduces spontaneous metastatic spread, maximal coverage would encompass the entire period during which there would be tumor emboli. This would extend from some variable time following tumor inception to the point at which any cells embolized by surgical manipulation had left the circulation or had been destroyed. Previous study has documented the time of initial metastatic spread in the tumor systems under study here, amputation prior to the sixth day following tumor inoculation being curative in virtually every case in both tumor

Table 4

<table>
<thead>
<tr>
<th>Groups</th>
<th>Operative deaths</th>
<th>Recurrence of tumor</th>
<th>Died free of metastases</th>
<th>Died with metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amputated only</td>
<td>0</td>
<td>5 (8%)</td>
<td>3 (5%)</td>
<td>34 (52%)</td>
</tr>
<tr>
<td>Pre-, intra-, and postamputation anticoagulants</td>
<td>7 (12%)</td>
<td>4 (7%)</td>
<td>5 (8%)</td>
<td>9 (15%)</td>
</tr>
</tbody>
</table>

Mortality of combined coumadin-amputation treatment of mammary adenocarcinoma in C3H/HeN mice.

*Difference significance of $P < 0.005$, $X^2$ test.
systems (5, 14). Tumor cell embolization, which increased with surgical manipulation, has been reported in both experimental animals and man (2, 13). The length of time following surgery that emboli continue to circulate is not exactly known, but following intravenous tumor cell injection in rodents, recovery of tumor cells from the circulation rarely extends beyond five hours (2). Thus the regimen of Warfarin therapy used in this study attempted to provide optimal coverage extending continuously from before the onset of metastatic spread, through surgery, and from 48 to 72 hours beyond. Coverage was not altered to diminish the likelihood of complications. The Warfarin therapy was very comparably effective against the metastatic spread from each of the tumors, and the difference between the two tumors in improvement of the cure rate would not appear to be due to a difference in the effectiveness of therapy in retarding metastatic spread. This comparable effectiveness of Warfarin therapy in reducing the metastatic spread from the different tumors, as well as its demonstrated effectiveness in reducing metastases in a wide variety of other animal host-tumor systems (21), suggests that it may influence some aspects of the metastatic process common to many malignant tumors. Since each clinical malignancy may be a biologically different host-tumor system, an effect against a common mechanism of metastasis formation could be advantageous.

The hope that these effects of Warfarin therapy, established by animal experimentation, may ultimately be of practical value is fostered by the retrospective study of Michaels (8), which suggests that anticoagulation may also favorably influence the metastatic spread of human cancer. The incidence of cancer was unchanged among patients receiving anticoagulant therapy for various thromboembolic diseases, but the metastatic spread was much less and the survival was better than would have been predicted statistically. Such suggestive data gives plausibility to the thought that, in human tumors, anticoagulation might be an effective adjunct to amputative therapy and most effective against tumors with a high rate of treatment failure due to metastatic spread.

REFERENCES

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