Induction of Acute Necrosis in Walker 256 Tumors in Rats

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SUMMARY

The destructive effect on cells of locally injected hypertonic solutions, resorption of which is delayed by some vasoactive agents, was observed in the skin and subcutis of the rat by Selye and his coworkers, who described it as acute conditioned necrosis. The same phenomenon was demonstrated in Walker 256 tumors implanted in the thighs of female Sprague-Dawley rats. A strongly hypertonic solution of glucose was injected once in and around the tumor (when it had reached a mean diameter of 1.5 cm), and 5-hydroxytryptamine was given s.c. at a distance. This resulted in disappearance of the growth in an average of 10 days and absence of metastases 2 months after treatment in 84% of animals as opposed to a spontaneous regression of the tumor in 4% of control rats.

INTRODUCTION

The vital importance for cells to bathe in an extracellular fluid of stable osmotic pressure, and the efficient mechanisms involved in its maintenance, have long been recognized. With regard to the trophicity of malignant cells, it was tempting to speculate on the deleterious effect of any wide and prolonged departure of their environment from isotonicity. Selye et al. (21) provided much food for thought in this respect by their experiments on the phenomenon of ACN. Whereas a hypertonic solution of glucose, sodium chloride, or urea, given s.c. to rats, is well tolerated, the same agents accompanied by injection at a distance of a mast cell discharger or a mast cell product (serotonin or histamine) cause extensive local skin necrosis (15, 16, 19–21). Another series of experiments, showing that resorption of an i.c.-injected solution of phenolsulfonphthalein was markedly delayed in decreasing order by octapressin, serotonin, mast cell discharger or a mast cell product (serotonin or histamine) cause extensive local skin necrosis (15, 16, 19–21). Another series of experiments, showing that resorption of an i.c.-injected solution of phenolsulfonphthalein was markedly delayed in decreasing order by octapressin, serotonin, mast cell dischargers, and histamine (17, 18), offered a tentative explanation for the ACN phenomenon and suggested an approach to the contemplated production of acute necrosis of tumors. Interest in serotonin, already noted for its potency in the experiments mentioned above, was further increased by the reports of Cater et al. (4, 6–8) on the hypoxia induced in Walker 256 tumors by i.p. 5-HT.

MATERIALS AND METHODS

With a 17-gauge trocar, 3 fragments (1 mm) of a 7- to 12-day-old Walker 256 tumor were implanted in the quadriceps of day-old Walker 256 tumor were implanted in the quadriceps of the right thigh of female Sprague-Dawley rats weighing between 95 and 110 g. One hundred sixty-five rats, each of which developed a growth of 1.5 cm mean diameter within 10 days (average, 6 days), were divided into 8 groups and treated as outlined in Table 1 on the day when this diameter was attained.

5-HT (serotonin creatinine sulfate from Nutritional Biochemicals, Cleveland, Ohio) was administered under the skin of the left hemithorax at a dose level of 2 mg in 0.2 ml of distilled water.

Anhydrous glucose (Anachemia Chemicals Ltd., Montreal, Quebec, Canada) was injected at a concentration of 1 g in 1 ml of distilled water with a 25-gauge needle in (1 ml) and/or around (1.5 ml) the tumor. In the latter case, about 1.2 ml of glucose were injected under the skin covering normal tissues, in the immediate vicinity of the tumor, on the outer aspect of the thigh, and 0.3 ml was injected deep in the connective tissue of the inner aspect of the thigh in contact with the medial aspect of the growth. Due to the high concentration of the glucose solution and its stickiness, care was taken to keep the solution-containing bottle in a hot water bath and have a maximum of 5 ml aspirated in the syringe. The small gauge of the needle (No. 25) prevented rapid injection, specially into the tumor, and required a high pressure on the piston; but a larger gauge would have facilitated leakage of the solution out of the tumor. All glucose injections were done under ether anesthesia.

Following this single treatment, the animals were observed for 2 months, during which body weight, size of tumor, area of cutaneous necrosis, and ulceration were measured 3 times a week. Autopsies were performed on dead rats, and metastases were carefully noted. The same procedure was carried out on the survivors at the end of the 2-month observation period, after they had been killed with chloroform. Animals in good general condition, showing neither growth on the right thigh nor macroscopic metastases, were considered to be cured.

Histological tumor studies with hematoxylin-phloxine were undertaken on separate animal groups 6, 12, 24, 36, and 48 hr after the above treatments.

Statistical analyses were made with the Fisher-Yates exact test of significance for a 4-fold contingency table.

RESULTS

Skin Necrosis. As in the ACN experiments of Selye et al. (21), we observed that the combination of glucose (in and/or around the superficially situated tumor) and 5-HT (s.c. at a distance) was damaging to the skin overlying and immediately surrounding the growth. Cyanosis appeared first and was followed by necrosis which was obvious within 24 hr.
The average dimensions of the necrotic areas (in cm) were 1.6 x 1.2 (5-HT + glucose in), 3.5 x 2.7 (5-HT + glucose around), and 4.3 x 3.2 (5-HT + glucose in and around). The average delay in the appearance of the ulceration and its healing, measured in days following treatment, was: 13 and 23 (5-HT + glucose in), 16 and 27 (5-HT + glucose around), and 13 and 29 (5-HT + glucose in and around). In certain cases, we observed some necrotic material originating from the deeper parts of the tumor and appearing at the level of the cutaneous ulceration.

**Regression of Tumors.** Spontaneous regression of the implanted tumor and the absence of macroscopic metastases at the end of the 2-month observation period were noted in 1 of 25 control animals. As seen in Table 1, other groups showed a higher percentage of survivors. Whereas the results obtained in rats receiving 5-HT or glucose around the tumor are not statistically significant, the higher incidence of cure in other groups is supported by a p < 0.05 value. Glucose in and around the tumor was more active than when it was injected in or around the growth. 5-HT considerably enhanced the effect of hypertonic glucose; the best results were obtained when the latter was injected both in and around the tumor.

Among the 70 animals that were cured, the average delay for the macroscopic disappearance of the growth (in days) after treatment was: 13 (5-HT), 14 (glucose in), 13 (glucose around), 16 (glucose in and around), 13 (5-HT + glucose in), 11 (5-HT + glucose around), and 10 (5-HT + glucose in and around). In the lone control rat whose tumor had regressed spontaneously, disappearance of the growth was noted on the 22nd day after implantation.

**Progression of Tumors.** Macroscopic metastases were detected in 72 of 95 animals that had died from progression of the tumor. The structures most commonly involved were the mesentery and the abdominal aortic lymph nodes (43 and 40 rats, respectively). The average survival time of these animals was fairly uniform in the 1st 7 groups (from 34 to 43 days), but it went up to 59 days in the 8th group (5-HT + glucose in and around). Among the 3 rats that had received the latter treatment without success, 1, whose tumor went on growing, was killed on the 82nd day after implantation. By then, its tumor was 5.4 cm long and 4.3 cm wide. An anterior mediastinal metastasis was noted in this rat. The other 2 animals died on the 47th and 48th postimplantation days, respectively.

**Body Weight and Tumor Size.** Noncured rats in the 1st 7 groups attained a maximal body weight (averages in the various groups ranged from 218 to 250 g) between 1 and 3 days before their tumors reached a maximal size (from 5.4 x 3.7 cm to 6.2 x 4.5 cm) and 4 to 6 days before their death. In the 8th group (5-HT + glucose in and around), the animal that was killed on the 82nd day after implantation showed maximal body weight and tumor size on that very same day. The other 2 rats from this group died 3 and 5 days, respectively, after acquiring maximal body weight and 3 and 2 days, respectively, after their tumors had attained maximal dimensions. Plotting of the maximal body weights of control and unsuccessfully treated animals showed that such measurements were practically always higher than the average weight of normal rats of a similar age. Conversely, the average body weight (252 g) of cured rats at the end of the 2-month observation period following treatment was comparable to the weight of normal rats of the same age.

**Tolerance of Treatments.** Systemic tolerance of the various treatments was good. However, 2 rats died from unknown causes 2 and 5 days, respectively, after receiving 5-HT + glucose in and around the tumor. As such short intervals did not permit any observation of the behavior of the tumors, these 2 animals were not included in our statistics.

**Histology.** Malignant cells invaded the muscular fibers in control rats. There were numerous mitoses, of which a good number were atypical. Small foci of necrosis were scattered throughout the tumor tissue (Fig. 1).

Six hr after the injection of 5-HT at a distance, the tumors showed marked dilation of capillaries which were filled with RBC. After 12 and 24 hr, areas of degeneration and necrosis were noted. After 36 hr, these necrotic regions had increased 15 to 40%.

Six hr after glucose injection into the tumor, capillary dilation was similar to that observed with 5-HT. Thrombi were found in some arteries and veins. After 24 and 48 hr, the necrotic areas were comparable in size to those seen in 5-HT-treated rats. Hemorrhages were evident within these necrotic zones and at their periphery.

The lesions were more mild after injection of glucose around the tumor. In some areas, after 24 and 36 hr, the capillaries exhibited ruptured walls, and fibrin was present between the malignant cells. In certain regions, these tumor cells displayed some degree of cytoplasmic degeneration with occasional pyknosis.

Hemorrhagic foci and dilated capillaries were discernible 6 hr after glucose injection in and around the tumor. At 12, 24, and 36 hr, areas of necrosis were noted.

The changes caused by hypertonic glucose were strikingly accentuated when 5-HT (injected s.c. at a distance) was added to the local treatment. Six hr after the 5-HT + glucose (in) injections, marked edema completely dissipated the tumor cells from one another, leaving clear necrotic zones and at their periphery.

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Cured rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>5-HT (2 mg s.c.)</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Glucose (1 g in tumor)</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>Glucose (1.5 g around tumor)</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>Glucose (1 g in tumor + 1.5 g around tumor)</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>5-HT (2 mg s.c.) + glucose (1 g in tumor)</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>5-HT (2 mg s.c.) + glucose (1.5 g around tumor)</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>5-HT (2 mg s.c.) + glucose (1 g in tumor + 1.5 g around tumor)</td>
<td>19</td>
<td>16</td>
</tr>
</tbody>
</table>

* NS, not significant.
spaces. Several such intercellular spaces contained RBC and fibrin. After 12, 24, and 48 hr, large necrotic zones were accompanied by extensive hemorrhagic foci. Fibrin was present around clumps of RBC or between tumor cells. However, the degenerative process did not seem to have touched some areas in the peripheral part of the tumor or less frequently in its center.

Six hr after injection of 5-HT + glucose around the tumor, edema was much less evident than in the previous experiment. However, after 12, 24, 36, and 48 hr, the necrotic areas were very extensive in some cases and occasionally involved five-sixths of the tumor. A few small tumoral foci appeared to be untouched by the degenerative process.

5-HT + glucose injection in and around the tumor produced changes similar to those caused by injection of 5-HT + glucose in the growth, although the necrotic areas were much more extensive (Fig. 2). A few tiny foci were usually found at the periphery of the tumor where there appeared to be intact malignant cells.

DISCUSSION

The working hypothesis of the deleterious effect on tumor cells of a hypertonic solution seems to have been verified in our studies and invites comparison with the results on ACN (21). Similarities exist, for example, in both investigations (21). No cutaneous changes were noticed when 5-HT or hypertonic glucose was injected alone, whereas a combination of both agents elicited skin necrosis. The same potentiating or additive effect of 5-HT on the hypertonic solution was found at the level of malignant cells. However, vascular changes (vasodilation, thrombosis) and occasional necrosis of the tumor were observed when either 5-HT or glucose (in, or in and around) was injected alone. This would confirm that the vascular system of malignant growths is more fragile than that of the skin and subcutis (1–3, 10–14, 22).

Injection of the hypertonic solution into the connective tissue surrounding the tumor deserves special comment. In keeping with the ACN experiments (21), it does not cause much damage to the connective tissue or to the growth itself. However, because hypertonic glucose (s.c.) + 5-HT (at a distance) could, in the ACN studies, produce thrombosis in veins and venules, occasional hemorrhages, edema of the s.c. tissue, and rapid necrosis of the skin, we expected that the combination of 5-HT + glucose (around) would induce thrombosis of the small vessels going to and coming from the tumor. This seems to have been verified by the macroscopic and histological results obtained. On the basis of these criteria, we could anticipate that glucose, in and around the tumor, in combination with 5-HT would improve the “cure” percentages of 5-HT + glucose in (66%) and 5-HT + glucose around (71%). Indeed, we were successful in 84% of the animals.

The choice of hypertonic solutions seems to be more limited in our type of experiments than in the ACN investigations. Whereas hypertonic sodium chloride (200 mg in 2 ml of distilled water) is well tolerated when it is injected s.c., it is rapidly lethal when it is introduced into a tumor, probably because of its quick passage into the blood stream. Apparently, a highly viscous solution of a large-molecular product must be injected into a richly vascularized structure like a malignant growth in order to avoid rapid resorption.

The remarkable potentiating influence of 5-HT, reported by Crile (9) under different circumstances, is still a matter of conjecture. It might produce circulatory stasis and edema through constriction of venules, increased capillary permeability, hypotension, and thrombosis (5), thereby resulting in local hypoxia where the safety margin is already narrow. The passage of plasma through the capillary walls in an effort to dilute the hypertonic solution in the extracellular compartment may only augment local hematocrit and facilitate thrombosis in vessels the endothelium of which has already been damaged by hypoxia.

Although treatment with 2 substances normally present in the body is well tolerated and could be repeated, we decided to administer them only once to test the efficacy of the method. For a similar reason, we chose to implant the tumor in the thigh. Preliminary experiments had shown us that tumors implanted on the back, under the skin of the caudal part of the thorax, are usually mobile over the underlying rib cage, when their mean diameter is 1.5 cm. Therefore, it is relatively easy to inject glucose under the deep surface of such tumors, and this leads to more frequent and rapid necrosis. The high rate of success achieved with the treatment of such well-circumscribed neoplasms might have been misleading. We felt that a tumor implanted within a well-vascularized structure like the quadriceps, in close proximity to the inguinal lymph nodes, would be more reminiscent of conditions and therapeutic problems encountered in most spontaneous growths.

We do not know at this stage whether the results obtained with the Walker 256 tumor might be duplicated with other experimental neoplasms and much less with spontaneous ones. Having expressed such reservations, we feel that the influence on malignant cells of a prolonged osmotic disturbance of their environment deserves further investigation.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Control Walker 256 tumor 6 days after implantation in the quadriceps muscle of the right thigh. A few muscle fibers can still be seen. Two tumor cells are in mitosis in right upper corner. Hematoxylin-phloxine, × 400.
Fig. 2. Walker 256 tumor 48 hr after injection of hypertonic glucose in (1 ml) and around (1.5 ml) the tumor and s.c. injection of 5-HT (2 mg) away from the tumor. A few muscle fibers can still be seen below center. The tumoral tissue is disorganized and edematous, and most of the nuclei are pyknotic or absent. Hematoxylin-phloxine, × 400.
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