Effect of Verapamil on Malignant Tissue Blood Flow in SMT-2A Tumor-bearing Rats

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ABSTRACT

We investigated the influence of the calcium antagonist verapamil on malignant and normal tissue blood flow using 25-μm 113Sn-labeled microspheres. Isogeneic Wistar-Furth rats were inoculated with a metastasizing mammary gland adenocarcinoma (SMT-2A) in the hindlimb musculature and mammary gland. Verapamil was administered as an i.v. bolus via an external jugular vein catheter followed by a supplemental constant infusion with a Harvard infusion pump. Plasma verapamil levels were determined by high performance liquid chromatography, and heart rate and systemic blood pressure were monitored. Verapamil in concentrations of 100 to 200 ng/ml resulted in an approximate 50% increase in tumor blood flow compared to control levels (p < 0.001) regardless of the site of tumor implantation. These levels were not associated with a significant alteration in arterial blood pressure. These data suggest that verapamil in concentrations currently used in humans may provide a means of improving the delivery of chemotherapeutic agents to solid neoplasms and may also enhance the effectiveness of ionizing radiation treatment by increasing tumor oxygenation.

INTRODUCTION

In general, antineoplastic treatment modalities seek to exploit fundamental biological properties of neoplastic tissue vis-à-vis normal host tissue. Virchow (48) in 1863 and Thiersch (41) in 1865 used injection techniques to study the vascular supply of human neoplasms. They noted an extravagant production of capillaries in the stroma of carcinomata and their growth from the capillary bed of surrounding tissues. In the ensuing 100 years, investigators have used a number of techniques including morphological analysis of light and electron microscopic micrographs, transparent chambers enabling in situ observations of tumor vasculature, and in vivo measurements of tumor blood flow such as can be obtained using radioactive microspheres to gain a clearer understanding of tumor microcirculation.

Mattsson and Peterson (28) have reviewed recently the literature on the influence of vasoactive drugs on tumor blood flow. They noted that reports could be found of both increased, normal, or decreased sensitivity to vasoactive drugs in the tumor microvascular bed. It appears that in many instances the effect of systemically administered vasoactive agents on tumor microcirculation is indirect, i.e., the change in tumor blood flow resulting from changes in systemic blood pressure and in the resistance of surrounding normal microvascular beds rather than a direct effect on tumor vessels themselves.

Vasoactive agents are useful probes for studying tumor microcirculatory physiology and in addition may in the future enhance the efficacy of several presently available antineoplastic treatment modalities. Increasing tumor blood flow relative to normal tissue blood flow should provide for enhanced delivery of chemotherapeutic drugs as well as lessen the fraction of hypoxic cells. Since hypoxic cells are 2 to 3 times more resistant to X-irradiation than those which are oxygenated, a decrease in hypoxic fraction should improve the results obtained when using ionizing radiation to treat tumors. Conversely, decreasing tumor blood flow relative to surrounding normal tissue should enhance the effectiveness of hyperthermia treatment as hypoxic cells and/or cells at low pH exhibit increased sensitivity to heat (7).

Previous attempts to increase tumor blood flow with vasoactive compounds have been largely unsuccessful and have led to the hypothesis that tumor vessels exist in a state of maximal vasodilation. Gullino and Grantham (11), however, noted an increase in malignant tissue blood flow following the systemic administration of acetyl-β-methylcholine to tumor-bearing rats, and Suzuki et al. (39) have reported recently increased tumor blood flow following angiotensin II-induced hypertension. Verapamil was introduced in 1962 as a potent vasodilator and has served as a prototype of a new class of compounds known as calcium antagonists. These agents do not interact with any known membrane receptors but interfere with calcium influx across excitable cell membranes and inhibit excitation-contraction coupling (42). To our knowledge, there are presently no reports in the literature on the effect of calcium antagonists on tumor microcirculation. The purpose of the present study was to investigate the influence of verapamil on malignant and normal tissue blood flow in rats bearing SMT-2A mammary adenocarcinomas.

MATERIALS AND METHODS

Animal and Tumor System. Female isogenic W/Fu rats weighing approximately 240 g were housed in groups of 3 to 4 in filter-top plastic cages with a bedding of wood shavings. They were placed in a temperature- and humidity-controlled room with 12 hr of light daily. Food and water were given ad libitum.

Tumor suspensions were prepared for transplantation with a Snell cytoseive as described previously (6) and were adjusted to a 10% volume of centrifugally packed cellular material from an SMT-2A mammary adenocarcinoma (21). Inocula of 0.10 ml were injected into both the right hindlimb musculature and right inguinal mammary gland.

Surgical Procedure. The animals were anesthetized with ether, and catheters were surgically placed into the left ventricle of the heart, the left femoral artery, and the right external jugular vein when tumors reached approximately 1 g. The animals were returned to cages and allowed to recover for approximately 3 hr before the experiments were performed.
performed. These procedures have been described in greater detail previously (19).

**Blood Flow Estimation.** Each experimental animal received, via the external jugular vein, a bolus injection of verapamil (isotop; Knoll Pharmaceutical Co., Whippany, N. J.) varying from 0.3 to 1.0 mg/kg body weight diluted in 0.9% NaCl solution to bring the injection volume to approximately 0.5 ml. To achieve higher drug concentrations, some animals were given supplemental constant infusions of verapamil (<120 μg/kg/min) with a Harvard infusion pump (Harvard Apparatus Co., Inc., Natick, Mass.). Control animals were given a bolus of 0.5 ml 0.9% NaCl solution by the same route. Approximately 15 min following the initial bolus, an Edco withdrawal pump (Edco Scientific, Inc., Chapel Hill, N. C.) was used to withdraw a femoral arterial reference blood sample for 1 min at a rate of 0.51 ml/min. Simultaneously, approxi-
mately 85,000 113Sn-labeled 25-μm microspheres (New England Nuclear, Boston, Mass.) were slowly flushed into the left ventricle with 0.5 ml of 0.9% NaCl solution. The reference blood sample was washed into a wide-mouth γ-counting vial for radioactivity analysis. Following completion of the experiment, the animals were sacrificed by an intracardiac infusion of 40 mg of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, Ill.). The animals were autopsied, and tissues of interest were dissected free of surrounding extraneous tissues, weighed, and placed in γ-counting vials. For each sample, the isotope activity and the corresponding number of spheres were determined by appropriate data reduction of the output from a 3-channel NaI well counter equipped with pulse height analyzers.

**Verapamil Plasma Level Determination.** Two min following the withdrawal of the microsphere reference sample, a 4-ml blood sample was collected via the femoral artery catheter and placed in a silanized vial with 50 μl of heparin (The Upjohn Company, Kalamazoo, Mich.). The animals were killed, and the plasma was stored and frozen in a silanized vial until further analysis. Plasma verapamil levels were determined by high-performance liquid chromatography as described by Harapat and Kates (12).

**Blood Flow and Resistance Calculations.** Tissue blood flow was calculated by the equation

\[
F_r = \frac{WR}{N_b \times N_f}
\]

where \(F_r\) is the tissue blood flow (ml/min/g), \(WR\) is the withdrawal rate of the integrated arterial blood sample, \(N_b\) is the number of spheres in the withdrawn blood sample, and \(N_f\) is the number of spheres per g of sample tissue. The systemic arterial blood pressure was measured with a Miller pressure transducer (Miller Instruments, Inc., Houston, Texas). A Gould electrocardiograph-rate preamplifier was utilized to determine the heart rate, and both the systemic pressure and heart rate were recorded on a Gould 4-channel recorder (Gould, Inc., Cleveland, Ohio). Assuming the mean venous pressure is equal to zero, the tissue vascular resistance (\(R_t\), mm Hg/ml/min/g) can be calculated by the equation

\[
R_t = \frac{P_s}{F_r}
\]

where \(P_s\) is the mean systemic arterial blood pressure (mm Hg) at the time of microsphere injection. Cardiac output (CO) was obtained from the equation

\[
CO = WR \times \frac{N_f}{N_b}
\]

where \(N_f\) is the number of spheres injected into the left ventricle. Total peripheral resistance (TPR) was calculated from the equation

\[
TPR = \frac{P_s}{CO}
\]

**Statistical Analysis.** The standard unpaired t test assuming unequal variances was used to compare blood pressure, heart rate, cardiac output, total peripheral resistance, blood flow, and resistance data for the control group to the corresponding values for verapamil-treated animals whose plasma verapamil levels fell within a given concentration range. A paired t test was used to compare differences in tumor blood flow for the 2 implantation sites within a given animal. Trends were also analyzed using linear regression techniques. All parametric statistical tests involving blood flow and resistance data were performed after natural logarithmic transformation, since this has been shown to adequately normalize the data (19).

**RESULTS**

Ratios of wet to dry tumor weights were obtained for control and verapamil-treated animals, and an absolute difference of less than 2% was observed between the 2 groups. All results given are therefore based on wet tumor weights.

Tumor blood flow data for the 2 implantation sites were found to be highly correlated (\(r = 0.67, p < 0.001\)) with a linear regression analysis yielding a slope of 1.16, which was significantly different from 1. The mean percentage of difference comparing the tumor blood flow for the 2 implantation sites within a given animal was less than 4% and was not significantly different from 0 (\(p > 0.10\)). For these reasons, the resistance and blood flow data for the 2 sites were combined in Chart 1, A and B, respectively. This chart depicts the effect of verapamil on tumor blood flow resistance and tumor blood flow as a function of verapamil plasma concentration. The number of tumors analyzed in each group is given in parentheses in the corresponding bar on Chart 1. Three animals with plasma levels >300 ng/ml were excluded from analysis due to profound hypotension (<60 mm Hg) following administration of the drug. The range of plasma levels for the >300-ng/ml group included in the study was 347 to 987 (mean, 688). As can be seen from Chart 1, verapamil levels between 100 and 200 ng/ml were associated with the greatest increase in tumor blood flow, although the difference between this group and the 200- to 300-ng/ml group was not statistically significant (\(p > 0.10\)). Linear regression analysis of all points <300 ng/ml revealed that both tumor blood flow and tumor vascular resistance were significantly correlated with verapamil plasma level (\(p < 0.001\)).

In Table 1, the malignant and normal tissue blood flow data for the control group and the 100- to 200-ng/ml group are compared. Among the normal tissues, only the skin overlying the tumor and the lungs exhibited statistically significant increases in blood flow (\(p < 0.05, p < 0.01\), respectively). Both the muscle- and mammary gland-implanted tumors exhibited statistically significant (\(p < 0.001\)) increases in blood flow of approximately 50%. The mean weight for the mammary-implanted tumors was 1.6 g for controls and 1.1 g for the 100- to 200-ng/ml animals (\(p > 0.10\)). The mean weight for the muscle-implanted tumors was 1.2 g for controls and 0.8 g for the 100- to 200-ng/ml group (\(p > 0.10\)). Jirtle (17) has demonstrated previously that blood flow to the SMT-2A tumor is independent of tumor weight over the range of tumor weights used in this study. Similarly, a linear regression analysis of tumor blood flow versus tumor weight for verapamil-treated animals yielded a slope of −0.01, which was not significantly different from 0 (\(p > 0.10\)). For both the control group and the 100- to 200-ng/ml verapamil treatment group, the mean number of microspheres contained in the reference arterial blood...
samples and in the tumors exceeded 400 so as to minimize random error (4).

Table 2 illustrates the effect of verapamil on systemic hemodynamic variables when plasma levels are between 100 and 200 ng/ml. In this concentration range, there was a significant ($p < 0.05$) fall in total peripheral resistance, while the mean arterial blood pressure remained essentially unchanged. The $t$ value for cardiac output was not significant comparing the 2 groups, but a positive correlation ($p < 0.05$) between verapamil concentration and cardiac output was demonstrated when a linear regression analysis on all points $<200$ ng/ml was performed. Heart rate tended to decrease ($p < 0.10$) indicating an increase in stroke volume.

**DISCUSSION**

The results from our investigation demonstrate that verapamil in concentrations from 100 to 300 ng/ml is capable of lowering tumor vascular resistance and increasing tumor blood flow. Calcium antagonists such as verapamil do not affect any of the known membrane receptors but do interact with a binding site for calcium ions (42). The depolarization of an excitable cell is caused by the inward current generated by the influx of sodium and calcium ions. Ordinarily, the sodium system is faster and quantitatively more important than the smaller and slower calcium current, which contributes to the net inward current chiefly during the plateau phase of the action potential (42). In addition to inhibiting excitation, calcium antagonists relax smooth muscle by reducing the calcium-dependent activation of myofibrillar ATPase (42). Verapamil preferentially acts on vascular smooth muscle undergoing phasic rather than tonic mechanical activity (10) and thus is about 20 times more effective in dilating arterioles than veins (34).

Malignant tumors appear to derive their vascular bed from newly formed vessels and by incorporation of existing vessels in the host tissue (28). Willis (49) has noted that the walls of arteries exhibit striking immunity from neoplastic invasion. During neoplastic growth, the development of the venous and capillary system exceeds that of the arterial system (16, 25). The newly formed vessels are of a capillary or embryonic type and are often dilated. Verapamil is capable of lowering tumor vascular resistance and increasing tumor blood flow in concentrations of 100 to 300 ng/ml, thereby improving the delivery of oxygen and nutrients to the tumor cells.
character, and they are usually very thin walled as compared to the diameter of their lumens (26). It has been repeatedly observed that tumor vessels are generally lacking in smooth muscle and adrenergic innervation (22–26, 30, 31). Tumor blood vessels necessary for regulation of the blood velocity, the direction of the flow, the increase of the blood flow capacity, and the regulation of the arterial pressure do not appear to develop (25). Papadimitriou and Woods (31) found recognizable arterioles and venules only in the peripheral 1 to 2 mm of transplantable neoplasms.

A review of methods for determining tumor blood flow has appeared recently (2), and the radioactive microsphere method introduced by Rudolph and Heymann (36) has been reviewed extensively (14, 15, 29, 33, 38). One of the requirements for studying cardiac output distribution with this method is irreversible trapping of the microspheres (15). The trapping of 15-µm spheres in normal capillaries has been shown to be virtually complete (9). Spheres passing arteriovenous shunts are trapped in the pulmonary capillary bed, preventing recirculation (20). The presence of multiple arteriovenous communications in tumors (9, 32) as well as the increased caliber of tumor vessels may act as a potential source of error, which may require the use of microspheres greater than 15 µm in diameter (19) as well as an estimation of the trapping of microspheres within the pulmonary microcirculation due to shunting.

One of the drawbacks of using the radioactive microsphere technique to study the influence of vasoactive compounds on tumor blood flow is the inability to define the precise anatomical location(s) at which a particular drug is acting. For example, the tumor vascular resistance one measures with this technique is actually the sum of all the resistances in series interposed between the efferent artery and the tumor capillary bed. For this reason, it is difficult to discern the relative contributions of changes in surrounding normal vessels, both in series and in parallel to the tumor, vis-à-vis changes in tumor-derived vessels, to alterations in tumor blood flow. The analysis of such local factors is further complicated by systemic perturbations, such as changes in cardiac output and blood pressure.

Nevertheless, it is still possible to put forth several hypotheses as to why tumor blood flow should exhibit increased sensitivity to calcium antagonists compared with normal tissues. In the heart, the sodium system develops embryonically later than the calcium system, and it is claimed that it is likely to be the first lost when cells are endangered and have reason to restrict their activity (42). If excitable cells such as smooth muscle cells are characterized by similar ontogenetic development, then one might predict, based on the embryonic appearance of tumor neovascularature cited above, that tumor vessels should exhibit increased sensitivity to calcium antagonists. Furthermore, increases in extracellular potassium inactivate the sodium system, again increasing the relative importance of the calcium system (42). In underperfused regions of solid tumors, one would expect an increase in extracellular potassium secondary to release of intracellular potassium from necrotic cells and/or intracellular-extracellular potassium shifts mediated by the accumulation of lactic acid.

It has been observed that sustained contraction of vascular smooth muscle can be evoked in the presence of low concentrations of endogenous vasoconstrictor substances when tissue anoxia develops and that this phenomenon can be blocked by the administration of calcium antagonists (44, 45). Vaupel et al. (46) using an oxygen microelectrode obtained a mean intratumor oxygen tension of 7 mm Hg in a C3H mouse mammary adenocarcinoma. In addition, hypoxia depresses glycolytic production of ATP in RBC which leads to diminished calcium ion efflux. As a result, the intracellular calcium content of RBC increases which leads to decreased deformability of the RBC membrane (35, 36). This decreased deformability increases the apparent viscosity of the blood and thus resistance to flow increases. The decrease in deformability combined with lowered blood flow favors rouleaux formation which also contributes to an increase in resistance to flow (45). Several calcium ion antagonists have been shown to counteract hypoxia-induced decreases in RBC deformability (6).

Finally, the peripheral vasodilation caused by calcium blockers leads to a decrease in intravascular pressure with a resultant compensatory increase in neural sympathetic activity and adrenal catecholamine release (1). For this reason, the effect of verapamil on normal host vessel resistance is attenuated by adrenergically mediated vasoconstriction, whereas the effect on tumor vessels is relatively unopposed due to their lack of adrenergic receptors.

The literature regarding the systemic hemodynamic effects of verapamil is somewhat contradictory due to disparities in the animal model chosen, the dosage and regimen used, and the degree to which the adrenergic system was perturbed by anesthesia and surgical implantation of monitoring devices. Our findings of increased cardiac output and decreased total peripheral resistance are consistent with the findings of Angus et al. (1) using anesthetized dogs and Vincenzi et al. (47) who studied healthy adults. These investigators, however, noted an increase in heart rate, while we observed a tendency for the

### Table 2

Effect of plasma verapamil (100 to 200 ng/ml) on systemic hemodynamic variables

<table>
<thead>
<tr>
<th>Blood pressure (mm Hg)</th>
<th>Total peripheral resistance (mm Hg/mL/min/g)</th>
<th>Cardiac output (ml/min)</th>
<th>Heart rate (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mean 93.3, 95% confidence interval 84.8–101.7</td>
<td>Mean 1.24, 95% confidence interval 1.09–1.42</td>
<td>Mean 74.7, 95% confidence interval 66.4–84.2</td>
</tr>
<tr>
<td>Verapamil (100–200 ng/ml)</td>
<td>Mean 90.9, 95% confidence interval 83.9–97.9</td>
<td>Mean 0.99, 95% confidence interval 0.87–1.13</td>
<td>Mean 90.8, 95% confidence interval 76.2–108.4</td>
</tr>
</tbody>
</table>

*p < 0.05, *p > 0.10

\[ n, \text{ number of observations.} \]
heart rate to decrease for plasma levels of verapamil at which we observed maximum tumor blood flows. The biphasic response we observed for tumor blood flow and cardiac output is similar to the biphasic response observed for contractility and heart rate by Angus et al. (1). He proposed that at lower doses reflex sympathetic stimulation predominates, while at higher concentrations the direct myocardial depressant effect assumes greatest importance. Mangiardi et al. (27) studying anesthetized open chest dogs found that verapamil levels less than 150 ng/ml produced few hemodynamic changes, while plasma levels exceeding 400 ng/ml were associated with marked decreases in arterial pressure, heart rate, peripheral resistance, and cardiac output. The calcium antagonist nifedipine warrants investigation, as it has been found in isolated tissue preparations following equimolar administration to be 12 times more potent than verapamil as a vasodilator (37) and has far less myocardial depressant effect (13).

Although previous attempts to increase tumor blood flow with vasoactive agents have been largely unsuccessful, there have been several reports in the literature of improving tumor blood flow through such means. Suzuki et al. (39) have described recently a 5.7-fold increase in tumor blood flow measured with an electric thermocouple when anesthetized rats bearing s.c.-transplanted AH109A solid tumors (Yoshida ascites hepatoma) were treated with angiotensin II, elevating their mean arterial pressure to approximately 150 mm Hg. In contrast, Jirtle et al. (18), using the radioactive microsphere technique to study tumor blood flow in unanesthetized, minimally disturbed rats bearing MT-W9B mammary adenocarcinomas treated with angiotensin II with similar blood pressure elevation, noted a decrease in tumor blood flow associated with a 3-fold increase in tumor vascular resistance. The ratio of tumor blood flow to surrounding normal tissue blood did, however, increase. The effect of angiotensin II on tumor blood flow is likely to depend on both the choice of tumor and the site of implantation. The vasoconstrictive action of angiotensin II consists of 2 components, namely a direct action on vascular smooth muscle and an indirect action mediated by increased sympathetic activity, the relative importance of which is dependent on species, vascular bed, route of injection, and dose (8). The vasoconstrictor effect of angiotensin II is strongest in the vessels of the skin, splanchnic region, and kidney, and blood flows in these regions fall sharply (8). Suzuki et al., however, reported no change in normal tissue blood flow including skin using the electric thermocouple they utilized to measure tumor blood flow. The question of the safety of angiotensin-induced hypertension for patients with underlying vascular disease such as the elderly remains to be answered.

Gullino and Grantham (11) reported increases in malignant tissue blood flow approaching the 50% increase we observed with verapamil when tumor-bearing rats were given injections of 2 to 10 μg of acetyl-β-methylcholine. They transplanted tumors into the kidney or ovary so as to provide a vascular pedicle and measured tumor blood flow directly by weighing venous outflow and reinjecting the blood into the circulation. While to our knowledge these results have not been replicated, it is of interest that many of the hemodynamic effects of choline esters are similar to those of verapamil. Choline esters, however, dilate vascular beds due to the presence of muscarinic receptors, despite the lack of apparent cholinergic innervation of most vascular beds (40). The systemic administration of choline esters is associated with a number of toxic side effects, and the therapeutic use of methacholine in humans has been virtually eliminated due to the unpredictability of the intensity of response (40).

Of interest regarding the use of verapamil as an adjuvant to chemotherapy is the recent paper by Tsuruo et al. (43) which reported enhanced cytotoxicity of vincristine and vinblastine with verapamil in vincristine-resistant P388 leukemic cells both in vitro and in vivo. They postulated that verapamil interfered with a calcium-mediated drug efflux mechanism in these resistant cells. Depending on the ubiquity of this process as a means of acquiring drug resistance, this may prove to be another rationale for the administration of verapamil along with chemotherapeutic agents.

In summary, we have demonstrated increased tumor blood flow associated with a decrease in tumor vascular resistance in SMT-2A mammary adenocarcinoma-bearing, unanesthetized rats treated with i.v. verapamil. The increase in tumor blood flow was independent of the site of implantation. The maximum effect was seen at plasma levels well tolerated in humans. We were able to obtain the increase in tumor blood flow without a statistically significant change in systemic blood pressure or normal tissue blood flow with the exception of the skin overlying the tumor and the lungs. The apparent increase in blood flow to the lungs was probably the result of increased blood flow through arteriovenous communications in both the tumor and normal tissues. For this reason, the increase in tumor blood flow we observed may actually be slightly underestimated. We conclude that verapamil may provide a means of improving the delivery of chemotherapeutic agents to solid neoplasms and may also enhance the effectiveness of ionizing radiation treatment by increasing tumor oxygenation.

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