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Heterogeneous Oxygen Partial Pressure and pH Distribution in C3H Mouse Mammary Adenocarcinoma[1]

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ABSTRACT

Severe disturbances in microcirculation during advanced phases of tumor growth lead to restrictions of convective and diffusive transport. In addition, an inhomogeneous distribution of transport conditions develops, resulting in insufficient and heterogeneous substrate supply and an inadequate drainage of wastes.

Polarographic measurements of the local tissue oxygen tension (PO_2) using gold microelectrodes reveal that very low PO_2 values are prevalent in C3H mouse mammary carcinomas. The tissue PO_2 frequency distributions are shifted to low PO_2 values and limited in variability. The mean PO_2 value is 7 mm Hg. The median is 4 mm Hg, the modal class being 0 to 5 mm Hg. Within different microareas of the same tumor, pronounced heterogeneities exist.

Due to an elevated rate of lactic acid production and its subsequent inadequate removal, a severe tissue acidosis is evidenced in malignant tumors. For C3H mouse mammary carcinomas, most of the measured pH values are in the range of 6.4 to 7.1, the modal class being 6.7 to 6.8 (mean pH, 6.73; median pH, 6.75). Within different microareas of the same tumor, clear heterogeneities in the pH distribution do occur. Very low pH values (5.8 to 6.3) have been observed in large ulcerated tumors. In extensively necrotic areas, pH values even higher than the arterial pH could be detected.

INTRODUCTION

Distinct changes in the microvasculature pattern and in microcirculation during advanced phases of tumor growth lead to restrictions of convective and diffusive transport within the tissue of many solid tumors (for reviews, see Refs. 37 to 39 and 43). In addition, an inhomogeneous distribution of the transport conditions develops causing an insufficient substrate supply as well as an inadequate drainage of wastes.

In order to elucidate the effect of these microcirculatory changes on tumor cell microenvironment, measurements of the local tissue oxygen tension in microareas of the tumor tissue using gold microelectrodes as well as recordings of tissue pH values utilizing spear-type glass microelectrodes were performed in C3H mouse mammary carcinoma.

MATERIALS AND METHODS

The *in situ* studies were carried out on a total of 11 female animals 10 to 20 days after implantation of tumor cells of C3H mouse mammary adenocarcinoma into the hind leg. The tumors were obtained from the Radiobiology Division, Massachusetts General Hospital, Boston, Mass. (34). This is a syngenetic implantable tumor using solid tissue transplants that are inoculated s.c. into receiving mice (conventional caging and feeding with commercial pellet rations and water *ad libitum*). The mice were anesthetized with chlorpromazine-HCl (50 mg/kg i.m.; Thorazine; Smith, Kline & French Laboratories, Philadelphia, Pa.) and ketamine-HCl (40 mg/kg i.m.; Ketaject, Bristol Laboratories, Syracuse, N. Y.). Rectal temperature was maintained close to 37° by a thermostatically controlled heating pad.

During the experiments, supplemental doses of the drugs (10 and 8 mg/kg i.m., respectively) were administered as required to maintain superficial anesthesia.

Measurement of Tissue pH Values with a pH Microelectrode. The pH microelectrodes used were of the Hinke type as described by Hebert (16) and Hinke (17). In these spear-type glass microelectrodes, tip diameters as small as 1 μm or less could be formed to minimize tissue damage upon penetration. The complete sensing length was between 10 and 50 μm. The response time was less than 15 sec as measured with a Keithley Model 610 B electrometer. The pH microelectrodes were linear over a pH range of 4 to 9. The drift of the microelectrodes used in this series of experiments was less than 1% of the measured value per hr. The sensitivity of the needle pH electrodes was 49 to 52 mV/pH unit at 25°.

For electrode calibration, pH reference buffer solutions supplied by Scientific Products were used. During calibration, the buffer temperature was 34°. (This temperature corresponded to the mean tumor temperature during our measurements, the core temperature being 37°.)

For details concerning the fabrication of the outer insulating pipets (Corning glass, Code 0120), the fabrication of the inner pH pipets (Corning glass, Code 0150), the sealing of the pH pipet to insulating outer pipet, the filling with 2.5 M KCl solution, and testing and cleaning of the microelectrodes, see Hebert (16). The pH glass electrode used was highly specific and experienced minimal interference from other ions. Only in highly basic solutions, *i.e.*, extremely low hydrogen ion activity, did the effect of alkali metal ions, such as sodium, become apparent (8).

The reference electrodes used with the pH microelectrodes were constructed from Corning glass, Code 0120 (capillaries: outer diameter, 1.0 mm; inner diameter, 0.5 mm), pulled to a tip diameter of approximately 1 μm, and filled with 2.5 M KCl solution.

The microelectrodes were placed in electrode holders (Model EH-1 S; W-P Instruments, Inc., New Haven, Conn.), fixed in a Narishige micromanipulator, and then connected to a D.C. preamplifier (type MPA-6; Transidyne General Corp., Ann Arbor, Mich.).

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Measurement of Tissue PO_2 Values Using O_2 Microelectrodes. The O_2 microelectrodes used during these experiments were of the "gold in glass" type as described earlier (5, 6). The exposed gold tip was about 1 to 5 μm in diameter and was coated with Rhoplex (Rohm and Haas Co., Philadelphia, Pa.) as described previously (2, 4). Silver print (GC Electronics, Rockford, Ill.) was applied to the shaft of the microelectrode to within 10 to 20 μm from the measuring gold cathode. The chlorided silver print served as the reference electrode. The entire microelectrode, except for the lower 2 to 3 mm, was coated with 5-Minute Epoxy (Devcon; Devcon Corp., Danvers, Mass.).

The electronic circuitry to measure the polarographic current was provided by a Model 1201 chemical microsensor system (Transidyne General Corp.) at a polarizing voltage of −750 mV.

In some experiments, PO_2 microelectrodes like those described by Erdmann *et al.* (10) were used. They were constructed from glass-coated gold wires with an outer diameter of about 5 to 8 μm fabricated by Battelle-Institut (Frankfurt A Main, West Germany). The beveled and polished glass-coated gold wires were inserted into glass capillary pipets drawn out on a horizontal pipet puller (Narishige, Inc., Tokyo, Japan). The tip of the microelectrodes was first coated with Formvar in ethylene dichloride (concentration, 0.25%; E. F. Fullam, Inc., Schenectady, N. Y.) and then covered with Rhoplex. Silver print, serving as the reference electrode, and 5-Minute Epoxy were similarly used as described above.

The procedures for electrode calibration and "conditioning" were similar to those described earlier (4, 31). The electrodes were "conditioned" by placing them into 0.2 M KCl solution for 10 to 12 hr at room temperature in order to obtain a stable current reading. Afterwards, the PO_2 microelectrodes were inserted into the tumor tissue for at least 30 to 60 min to obtain a "tissue membrane" in front of the artificial Rhoplex membrane before calibrating in 34° 0.2 M KCl solution. This special treatment of the microelectrodes made it possible to read accurate tissue PO_2 values and to exclude negative PO_2 readings in the tissue. The validity of this technique has been proven in earlier publications utilizing other methods to obtain an insight into the oxygenation of tissues (40, 42). Thus, it is assured that the very low PO_2 values obtained in the tumor tissue are not simply related to the reduced oxygen conductivity of the tissue [at 37°, the diffusion coefficient D_{O_2} is 3.0 sq cm/sec for the 0.2 M KCl solution and 1.5 sq cm/sec for the tumor tissue (36)].

Pure nitrogen or glucose together with glucose oxidase in 0.2 M KCL solution were used for reading at zero oxygen tensions. The residual current at zero O_2 tension was very low and the response of the microelectrode to PO_2 changes was very rapid (mean 95% response time, 2.1 sec). The relation between current output and O_2 partial pressure was linear (average slope, 4×10^{-11} amp/mm Hg). The temperature dependence of the O_2 microsensor was 3.8% per degree. Due to the suitable membrane covering, the microelectrodes used were virtually insensitive to stirring.

The PO_2 microelectrode was also fixed in a micromanipulator. Pre- and poststudy calibrations of the pH and PO_2 electrodes were always performed, the time intervals of tissue measurements being 30 to 45 min.

The pH readings were evaluated only if prestudy and poststudy calibrations were identical. For PO_2 readings, the poststudy calibrations were within ±5% of prestudy calibrations (corrections for changes in sensitivity were made assuming linear changes with time). All experiments were conducted in an electrically shielded Faraday cage.

Experimental Protocol. After careful removal of the skin and of s.c. tissue, the electrodes which were in close approximation to each other were inserted to an initial depth of about 200 to 400 μm in order to avoid the influence of the atmospheric oxygen on the PO_2 readings in the tissue. Afterwards, the microelectrodes were further advanced in steps of 100 μm into the tumor tissue.

Steady-state readings of the measured parameters were taken from the recordings on a Grass Model 78 D polygraph. In each tumor, 1 to 4 microelectrode tracks were performed. In order to measure the relevant parameters of respiratory gas exchange under these conditions, micro blood samples were taken from the abdominal aorta after the PO_2 and pH recordings were performed (IL type 213 blood gas analyzer).

PO_2 and pH Measurements at the Site of Implantation in Healthy Mice. In order to have control measurements, PO_2 and pH readings were performed in s.c. tissue layers and in superficial muscle layers in healthy animals (n = 6). In these experiments, measurements were achieved by advancing the microsensors from an initial depth of 0.4 to about 2 mm.

Evaluation of the Results and Statistics. To gain an insight into the intratumor pH and PO_2 distribution, relative-frequency histograms of the measured values were compiled. In the case of the PO_2 histogram, the measured tissue oxygen tensions were grouped into classes of 5-mm Hg intervals. Intervals of 0.1 pH units were used for pH histograms. Means ± S.D. of the data were reported throughout.

RESULTS

PO_2 Distribution in C3H Mouse Mammary Carcinoma. Polarographic measurements of the local tissue oxygen tension (PO_2) in microareas of the tumor tissue revealed that, in most tumor areas, the PO_2 profiles were quite uniform, and a monotonous pattern of very low PO_2 values predominated. Greater regional PO_2 differences as well as higher mean PO_2 values could be found only in tissue areas where a sufficient nutritive blood flow existed. The PO_2 gradients were almost completely uniform and very flat. This was in contrast to most of the normal tissues where steeper gradients can be found.

The typical feature of the tissue PO_2 distribution is illustrated by the PO_2 histogram in Chart 1. Taking into account 2912 PO_2 readings within 11 tumors (mean tumor wet weight, 0.50 ± 0.37 g), there was a shift of the O_2 partial pressure distribution to lower values, *i.e.*, the frequency distribution was tilted to the left and more limited in variability than in normal tissues at the site of implantation. Considering 346 PO_2 readings in healthy mice, the PO_2 values measured were in the range of 4 to 82 mm Hg; the mean O_2 partial pressure was 34 mm Hg. Under these experimental conditions of normal respiratory gas parameters within the arterial blood, the median was 31 mm Hg, the modal class being 25 to 30 mm Hg for s.c. tissue layers and superficial muscle layers before tumor implantation.

Since more than 50% of the measured values were in the range of 0 to 5 mm Hg, there was clear evidence that tissue hypoxia is a common feature in C3H mouse mammary carcinoma.

This peculiarity is particularly evident if the mean PO_2 values in the tumors are described as a function of tumor wet weight. Chart 2 shows that there was a significant logarithmic correlation between these 2 parameters.

There were pronounced heterogeneities within the same tumor looking at different microelectrode tracks. In Chart 3, the PO_2 distribution of 3 different tracks within the same tumor (wet weight, 0.64 g) are compiled. Chart 3 clearly indicates that, within the same tumor, distinct heterogeneities in the PO_2 distribution could be detected. This is mostly due to a heterogeneous distribution of blood flow in microareas of the tumor tissue (41). The relevant respiratory gas parameters in the arterial blood under these conditions were as follows: PO_2, 92 ± 18 mm Hg (1 mm Hg = 0.133 kPa); PCO_2, 27 ± 7 mm Hg; and pH, 7.29 ± 0.13.

pH Distribution in C3H Mouse Mammary Adenocarcinoma. As a result of an elevated rate of lactic acid production and its subsequent inadequate removal, a severe tissue acidosis develops in malignant tumors. In contradistinction to this finding, the pH values within the normal tissue at the site of implantation varied from 7.12 to 7.37. During normal acid-base status, the mean pH was 7.31. Taking into account 270 readings, the median was 7.30, the modal class being 7.3 to 7.4.

In Chart 4, a frequency distribution of measured pH values in 11 tumors (1453 pH readings) during normoxia and normoglycemia is presented. For C3H mouse mammary adenocarcinoma, very low pH values were the common feature. Most of the measured pH values were in the range of 6.4 to 7.1. The mean pH value under these conditions was 6.73. The median was 6.75, the modal class being 6.7 to 6.8.

The unusually low pH values at the left side of the histogram were the result of measurements in a large ulcerated tumor with a wet weight of 1.5 g (pH, 5.8 to 6.3).

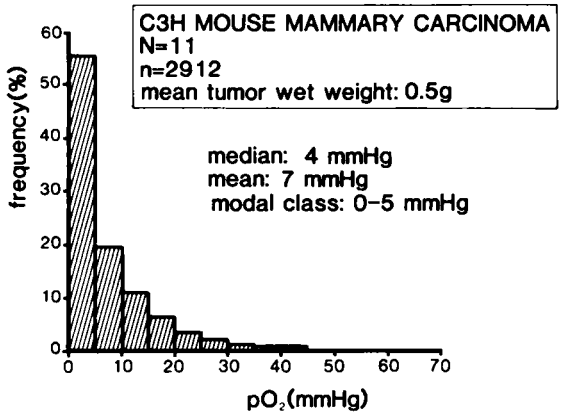

Chart 1. Tissue PO_2 frequency distribution (PO_2 histogram) in C3H mouse mammary adenocarcinoma. *N*, number of tumors, *n*, number of PO_2 readings.

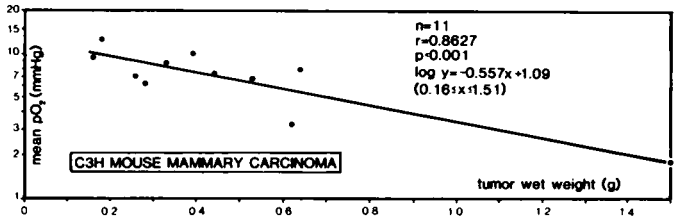

Chart 2. Mean tissue PO_2 values in C3H mouse mammary carcinoma as a function of tumor wet weight. *n*, number of tumors.

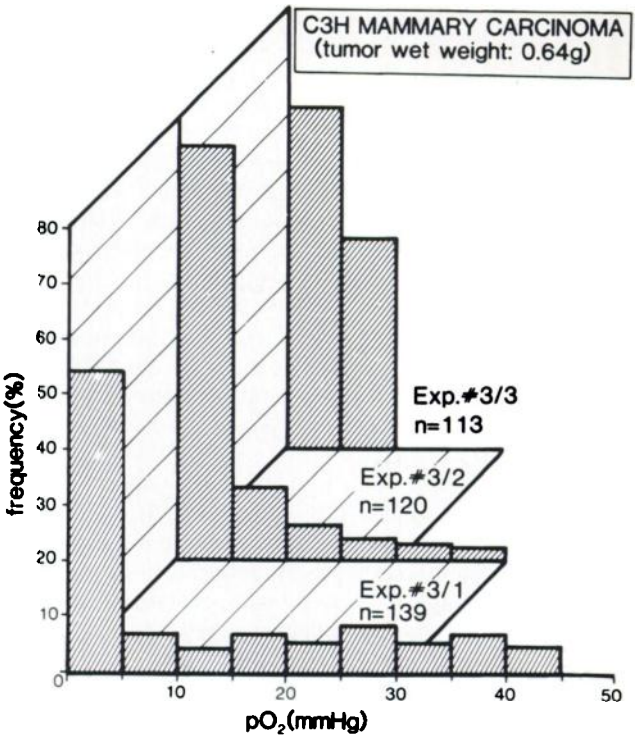

Chart 3. PO_2 frequency distributions of different microelectrode tracks within the same tumor, indicating distinct heterogeneities in the PO_2 distribution within malignant tumors. *Exp. # 3*/three electrode tracks; *n*, number of readings.

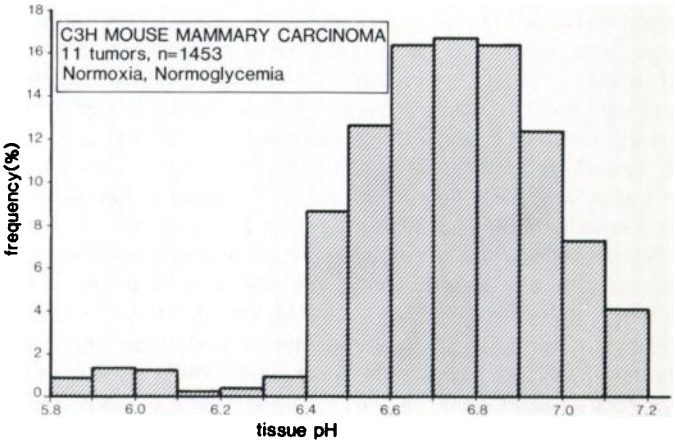

Chart 4. Frequency distribution of measured tissue pH values by means of spear-type glass microelectrodes (pH histogram). *n*, number of readings.

Usually, the measured pH values within one microelectrode track did not vary more than 0.4 pH unit. However, looking at different areas of the same tumor, marked heterogeneities in the pH distribution were observed. In Chart 5, the pH distributions within different microelectrode tracks are presented. The results substantiated the fact that pronounced variations in the pH distribution did exist.

During the pH measurements, no significant correlation between the mean tissue pH values and the tumor wet weight could be detected, as was the case with the tissue PO_2 values. This was due to the occurrence of pH values even higher than the arterial pH in larger tumors with extensively necrotic areas. During development of necrosis, the pH was acid while glycolysis of glycogen stores continued. Once the necrotic process

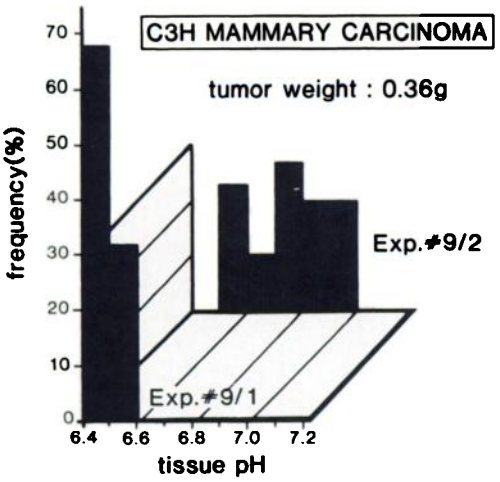

Chart 5. pH frequency distributions of different microelectrode tracks within the same tumor, indicating distinct heterogeneities in the pH distribution within C3H mouse mammary adenocarcinomas. *Exp. # 9*/two electrode tracks.

had been fully established and glycogen stores were exhausted, the pH became alkaline. This finding is supported by the fact that necrotic tissue is acidophilic and that calcium is frequently deposited in old necrotic areas which can occur only at high pH values.

DISCUSSION

During advanced phases of tumor growth, there are typical changes in the microvasculature pattern within the tissue of many solid tumors. A selection of the most relevant modulations is listed in Table 1. Partly due to these peculiarities of tumor vascularization, a series of characteristic variations of tumor microcirculation occurs (important details on this topic are listed in Table 2).

These alterations lead not only to marked restrictions and inhomogeneities of both convective and diffusive transport but also to an impairment of the efficiency of antitumor therapies which utilize antiproliferative agents and/or irradiation.

The poor oxygen supply and the inadequate removal of lactic acid are compulsory manifestations of the reduction of the terminal vascular bed as well as of the heterogeneities in microcirculation which become more extensively evident with increasing tumor size and tumor age, respectively.

Critical oxygen supply to the tumor tissue, tissue hypoxia in malignant tumors, and their impact on tumor growth have been discussed in detail (35, 37–39, 44, 45). In preceding publications, a critical valuation of PO_2 measurements in tumors has been presented (35, 37–39, 45).

The PO_2 distribution pattern in C3H mouse mammary carcinoma coincides very well with earlier results in tissue-isolated rat tumors (35, 37–39, 44, 45). At comparable body weight/tumor wet weight ratios, nearly identical PO_2 frequency distributions can be detected as shown in Chart 6.

Several experiments on mammary carcinoma in mice have shown that these tumors contain a significant proportion of hypoxic cells, often 20 to 25% (for reviews, see Refs. 15 and 23). Although the experimental plan used by most authors to determine the percentage of hypoxic cells, *i.e.*, radiobiologically hypoxic cells, is not comparable at all with the experimental tools used in our experiments, the results are quite compatible. Regarding only those experiments in which pre- and post-study equilibrations are identical, the PO_2 readings within the tumor tissue can be presented in the following classes which are based on radiobiological criteria (Table 3). The lowest class comprises values of 0 to 1 mm Hg and thereby represents the radiobiologically hypoxic fraction.[3] In the PO_2 range between 2 and 5 mm Hg, the radiosensitivity has not yet reached one-

Table 1

Microvasculature pattern of malignant tumors during advanced growth states

Absence of a sufficient neovascularization
Decrease of number of nutritive vessels per unit tissue volume
Reduction of vascular space per unit tissue volume
Decrease of vascular surface area per unit tissue volume
Widening of vessel diameter
Increase in vessel length
Broadening of intercapillary distances
Appearance of lacuna-like, sinusoidal, and cystiform blood vessels
Increase in number of arteriovenous anastomoses
Loss of hierarchy in vessel arrangement
Development of enlarged venular blood vessels
Emergence of fragile blood vessels
Crushing of vessels by tumor cells that proliferate rapidly into the limited space within the tumor
Distortion of blood vessels
Emergence of hemorrhage
Appearance of thin endothelia with wide gaps and lacking pericytes
Increasing heterogeneities in vascularization with increasing tumor mass

Table 2

Typical features of tumor microcirculation during advanced tumor growth states

Decrease of tumor blood flow per unit tissue volume
Increase of vascular flow resistances
Arteriovenous shunt perfusion
Vascular prestasis and stasis
Emergence of micro- and macrothromboses, occlusion of vessels
Absence of drainage of lacuna-like blood vessels
Existence of nonflowing capillaries
Occurrence of absolute ischemia in some tissue regions
Emergence of regurgitation and intermittent circulation
Unstable speed and unstable direction of blood flow
Appearance of distinct heterogeneities in microcirculation
Loss of RBC flexibility due to severe tissue acidosis
Disappearance of vasomotion and occurrence of physiologically unresponsive blood flow
Abnormal permeability of the vessel wall
Existence of marked rheological heterogeneities
Pronounced inhomogeneities in capillary hematocrit

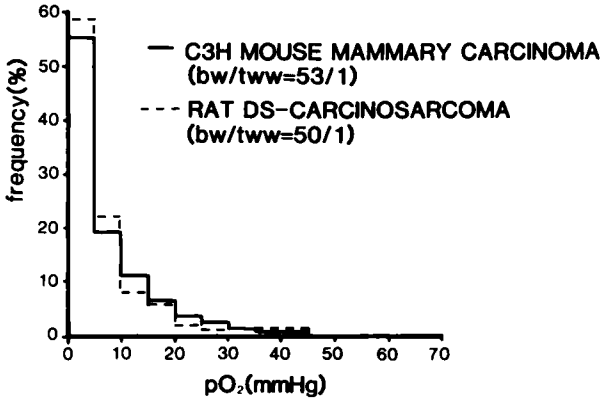

Chart 6. Comparative PO_2 histograms of different malignant tumors. The PO_2 frequency distribution of C3H mouse mammary carcinoma is compared with the PO_2 histogram from earlier measurements on DS carcinosarcoma in the rat kidney (35, 37–39, 44, 45) considering similar body weight/tumor wet weight ratios (*bw/tww*).

[3] R. M. Sutherland, personal communication.

half its maximum value at 37° (24). Above 6 mm Hg, the sensitivity is increasing and finally reaches its maximum value at PO_2 readings higher than 30 mm Hg (15, 24).

Considering the lowest class from Table 3 as the fraction of hypoxic cells, and taking into account earlier findings on the changes in the proportions of hypoxic cells after irradiation of transplanted mouse mammary tumors (18, 22, 33), the available data can be summarized in Chart 7, in which the percentage of hypoxic cells as a function of tumor wet weight is illustrated. The present *in vivo* data, utilizing polarographic PO_2 measurements together with literature data, clearly show that there is a significant correlation between tumor wet weight and percentage of hypoxic cells in mouse mammary carcinoma.

As already mentioned above, the radiosensitivity of tumor cells reaches one-half its maximum value at 37° if the oxygen tension is about 5 mm Hg (24). Considering this dependence of radiosensitivity on oxygen partial pressure and on oxygen concentration, respectively, the conclusion can be drawn that, with increasing tumor wet weight from 0.21 to 0.33 and 0.62 g, the radiosensitivity of 50, 59, and 77% of the tumor cells is already less than one-half the maximum value. In addition, as a heterogeneous PO_2 distribution is a general pattern of many solid tumors, the radiosensitivity must be unevenly distributed over the tumor mass.

The insufficient and heterogeneous oxygen supply yields a low and inhomogeneously distributed oxygen consumption of the tumor tissue. Some recently developed techniques rendered it feasible to measure the O_2 consumption with a much finer resolution even in microareas and thereby to relate variations in the oxygen uptake with distinct histological structures in the tissue. In a series of experiments, Constable *et al.* (7) were able to demonstrate a heterogeneous O_2 uptake in different malignant tumors in animals and humans. The heterogeneity has been related to different vascular structures and to regions of necrosis or cellular differentiation. The variations of the O_2 uptake values as described by these authors increased with enlarging tumor mass.

From simultaneous measurements of the PO_2 distribution within microareas of the tumor tissue and of the microflow distribution utilizing the hydrogen clearance technique (41), there is clear indication that the oxygenation of the tissue directly reflects the local efficiency of tumor blood flow. Usually high PO_2 values are associated with well-perfused tumor tissue areas, whereas the lowest PO_2 values could be found within most poorly perfused regions or within areas where no longer any flow could be detected. This inhomogeneity in tissue blood flow and in PO_2 distribution seems to be random. There is no clear evidence that the heterogeneities are somewhat structured in this type of tumor. [At special positions of the microelectrodes used, the tip location could be determined by staining the tissue with Alcian blue injected hydrostatically through a micropipet attached to the microsensor (for details, see Ref. 25).] Due to the thicker shaft, the position of the probes for the H_2 clearance technique was detectable through a series of subsequent tissue slices.

Table 3

Relative frequencies of tissue PO_2 values measured in tumors of different sizes

The PO_2 classes have been chosen according to radiobiological criteria (see text for detailed explanation). Tumor wet weights are as follows: small tumors, 0.21 ± 0.07 (S.D.) g; medium-sized tumors, 0.33 ± 0.06 g; and large tumors, 0.63 ± 0.03 g.

	% of frequency in tumors of following sizes		
PO_2 (mm Hg)	Small	Medium	Large
0 and 1	24.2	36.4	55.4
2 and 3	19.2	18.2	17.5
4 and 5	7.2	5.0	4.5
6–10	24.6	15.0	13.1
>10	24.8	24.4	9.5

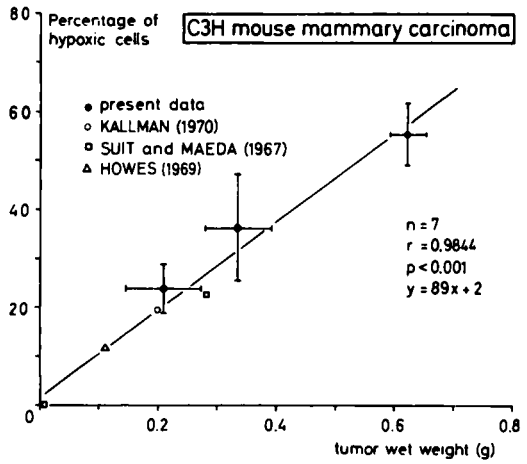

Chart 7. Percentage of hypoxic cells as a function of tumor wet weight (mouse mammary carcinoma). This analysis is based on literature data utilizing theoretical survival curves (18, 22, 33) as well as on tissue PO_2 measurements and considering that PO_2 values of 0 to 1 mm Hg represent the radiobiologically hypoxic fraction. Values are means. *Bars*, S.D.

A great number of investigations has unequivocally shown that pH values within the tissue of malignant tumors are lower than those of normal tissues (1, 3, 9, 14, 20, 21, 26–28, 30, 32, 46–49).

During most of these experiments, pH-sensitive glass electrodes have been used. With the exception of von Ardenne *et al.* (46–49), only quite large pH electrodes have been applied on malignant tumors. However, inserting pH electrodes with large-tip diameters must cause severe tissue damage and, consequently, disturbances of tumor regional blood flow. In addition, the spatial resolution of those large electrodes is very poor and, therefore, only measurements integrating the pH values of larger tissue areas could be performed. Utilizing pH microelectrodes with tip diameters of 1 μm, it is possible to prevent extensive disturbances within the tissue during the period of observation. Since microelectrodes with very small tips were used in the experiments presented, the spatial resolution is distinctly improved, thus enabling pH distribution measurements in microareas of the tumor tissue. At this point, it should be emphasized that there are results suggesting a more basic (alkaline) tissue milieu in tumors than in normal tissues (11–13, 19, 29). Unfortunately, it cannot be excluded that these findings result from methodological problems or that tumors with extensively necrotic areas were used.

The severe tissue acidosis in the tumor tissue causes a series of deleterious effects. Besides affecting a number of biochemical processes, low pH values induce a considerable stiffening of the RBC membrane and, hence, lead to further deteriorations of tumor microcirculation. Furthermore, the loss of flexibility of the RBC membrane leads to an inhibition of the convective transport of oxyhemoglobin and oxygen within the RBC and thereby impedes the oxygen release from the erythrocytes to the tissue (50). Because there are pronounced

heterogeneities in the pH distribution within different microareas of the same tumor, greater heterogeneities in the O_2 release pattern have to be expected.

Disregarding the very low pH values which were present during measurements in a large ulcerated tumor, there was no significant correlation between the microflow within the tissue and the pH values obtained. This is partially due to the finding that, in no-flow areas, the pH can be low (during ischemia) or even elevated (in necrotic areas). This is in contradistinction to the PO_2 findings, which can be used as a relevant mean for the comprehension of the local flow conditions.

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