A NEW CONCEPT OF THE FORMATION OF HYPOXIC CELLS IN TUMORS AND ITS POSSIBLE IMPLICATIONS FOR RADIOTHERAPY AND CHEMOTHERAPY

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SUMMARY

Hypoxic cells in tumors are proposed to consist of at least 2 types, depending on whether they remain hypoxic for long (chronic hypoxia) or short (acute hypoxia) periods. Experimental evidence of the possible presence of acutely-hypoxic cells in one type of murine tumour is presented. Finally, the possible implications for radiotherapy and chemotherapy of the presence of acutely-hypoxic cells in human tumors is discussed briefly.

INTRODUCTION

Hypoxic cells are much less sensitive to killing by ionizing radiation than well-oxyenated cells. This is the so-called "oxygen effect" that radiobiologists and radiotherapists are familiar with. The presence of hypoxic cells in many types of human and rodent solid tumors is now well established (Hall, 1978; Moulder and Rockwell, 1980). It has often been suggested that these hypoxic cells may survive a treatment with ionizing radiation because of their low radiosensitivity. Following the treatment, the surviving cells may then repopulate the tumor.

Because of their possible relevance to radiotherapy, it is obviously of great importance to understand how hypoxic cells arise in tumors. By understanding more about this aspect of hypoxic cells, it is hoped that we will learn how to deal more effectively with them. The usual explanation cited for the existence of hypoxic cells is based on the inability of oxygen to reach cells far away from a blood vessel. In this article, I shall present evidence based mainly on my research at the Ontario Cancer Treatment and Research Foundation Victoria Hospital, Canada and the Department of Biophysics, University of Western Ontario, Canada which have raised some interesting questions about the adequacy of this simple explanation for the existence of hypoxic cells in all types of tumors.

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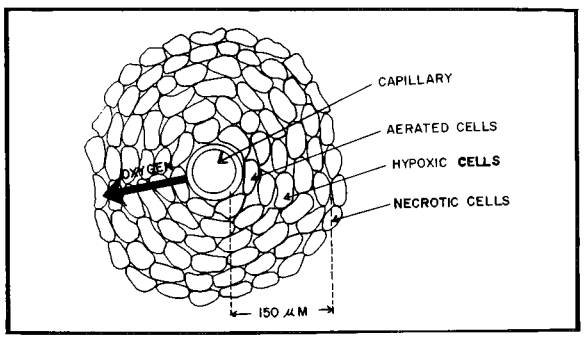

Fig. 1. Diffusion of oxygen from a blood vessel supplying oxygen and nutrients to a tumor tissue.

HYPOXIC CELLS IN TUMORS

Let us first briefly review the explanation usually cited for the existence of hypoxic cells in tumors. Oxygen diffusing outwards from a blood vessel supplying the tumor tissue is consumed by the tumor cells through respiration (Figure 1). The cells immediately surrounding the capillary receive an adequate amount of oxygen and are thus "well-oxygenated". The oxygen gets depleted as it diffuses further outwards and by about 150 μm from the capillary, "anoxic" cells are found. This distance represents the maximum diffusion distance of oxygen. Hypoxic cells are found between the "well-oxygenated" and the "anoxic" cells. They form a layer with a thickness of probably a few cell diameters. Perhaps the most

important experimental evidence supporting this explanation for the existence of hypoxic cells are the studies of Thomlinson and Gray (1955). In human bronchial carcinoma, they found that cords of tumor cells having a radius in excess of about 200 μm were accompanied by necrotic cells. No necrosis was seen if the radius was smaller than about 160 μm. By using the diffusion equation, they were able to estimate that a complete depletion of oxygen was expected at a distance of about 150 μm from the blood vessel. Their findings would appear to directly implicate a lack of oxygen as the cause of necrotic cells, and, indirectly, the presence of hypoxic cells in a human tumor.

BUOYANT DENSITY STUDIES

Part of my studies involved an attempt to physically separate and recover the hypoxic cells from a tumor in order to investigate their characteristics. For these studies, the EMT6 tumor cell line was used because it can be grown *in vitro* in tissue culture or *in vivo* as a solid tumor in mice (Rockwell *et al,* 1972). Tumors cells were cultured *in vitro* in an environment of different oxygen tension by growing them on tissue culture plates and keeping the plates in air-tight chambers. By evacuating the air from the chambers and replacing it with a gaseous mixture containing mainly nitrogen, the amount of oxygen present in the chamber could be easily measured and controlled. The cells were then harvested from the plates and their buoyant density was determined by isopycnic centrifugation (Ng *et al,* 1980a). Cells grown in hypoxic environment were found to be significantly lighter in density than control cells grown in a normal, oxygenated environment (Figure 2)

The buoyant density study was repeated for cells from the EMT6 solid tumor. The tumor tissue was digested by an enzymatic technique chosen to produce maximal recovery of the tumor cells (Ng and Inch, 1978). On the basis of the *in vitro* data, the buoyant density distribution of the solid tumor cells was expected to consist of at least a bimodal distribution, with the oxygenated and hypoxic cells being found at different densities. However, the result that was obtained was quite unexpected. A

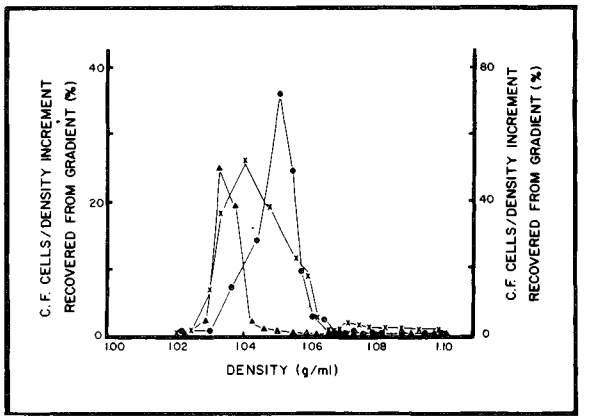

Fig 2. The density profiles of clone-forming (C.F.) cells grown at 37°C in medium equilibrated with air (about 2.1 x 10^5 ppm oxygen) or under hypoxic conditions (2.1 x 10^3 ppm oxygen) for 16 or 66 hours. The symbols used refer to:

	Oxygen (ppm)	Time (hours)
•—•	2.1 x 10^5	16
x—x	2.1 x 10^3	16
▲—▲	2.1 x 10^3	66

Ordinate on the left refers to (•—•) and (x—x) profile.
Ordinate on the right refers to (▲—▲) profile.
The abcissa is the same for all 3 profiles

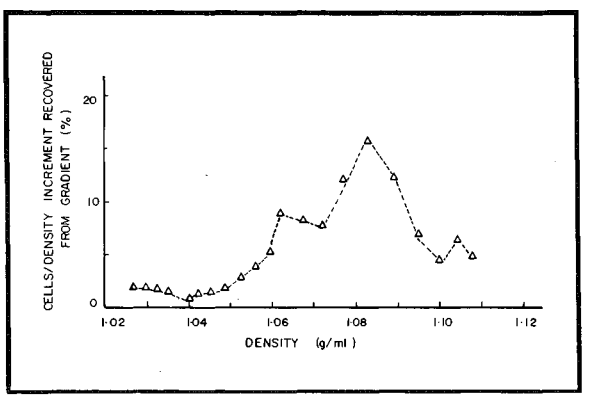

Fig. 3. The density profile of cells from a solid EMT6 tumor. The solid tumor was disaggregated into single cells using a combination of enzymatic - mechanical method before the buoyant density of the cells was determined.

rather homogeneous distribution was observed (Figure 3)

Since the hypoxic cells could not have become "oxygenated" while their buoyant density was being determined, the results of Figure 3 suggested that the hypoxic cells might still be present among the lighter portion of the density distribution but that

there were too few hypoxic cells for them to be seen as a distinct "peak". To investigate this possibility, the solid EMT6 tumor was treated with 2000 rad X-rays, a dose known to be large enough to kill most of the oxygenated but not the hypoxic cells, before the buoyant density of the cells was determined (Ng *et al,* 1980c). It was reasoned that the density distribution that followed would be essentially that of the surviving, hypoxic cells. In this manner, the buoyant density of the hypoxic cells from the solid tumor could be easily determined and compared to the *in vitro* data. However, when this was done, the density distribution of the surviving, hypoxic cells was not different from that of control cells from unirradiated tumors.

What do these results mean? It would appear that the *in vitro* and *in vivo* data were contradictory. Hypoxic cells *in vitro* were significantly less dense than non-hypoxic cells whereas hypoxic cells *in vivo* were not significantly different in density. The existence of radiobiologically hypoxic cells (cells at oxygen tension of less than 2000 ppm) in the EMT6 tumor has been established by previous studies (Rockwell and Kallman, 1973). There was, however, one possibility which may explain the *in vitro* and *in vivo* observations. As the buoyant density of a cell would reflect its overall chemical composition, it was thought that the cell must be exposed to a hypoxic environment for some time before change in buoyant density would occur. The hypoxic cells *in vitro* could have been exposed to such a condition whereas the hypoxic cells in the tumor might not.

To test the hypothesis that cells must be kept in a sustained hypoxic environment to develop a change in their buoyant density, the following experiment was designed. The tumor cells were grown *in vitro* in a specially-designed vessel and allowed to become hypoxic gradually as the oxygen in the culture medium was consumed through respiration (Ng *et al,* 1980b). This kind of hypoxic environment was thought to be perhaps more representative of how cells become hypoxic in tumors *in vivo* instead of the use of the air-tight chambers previously described. After the cells had become hypoxic, the oxygen tension was varied between 4000 ppm and less than 10 ppm. Interestingly, it was found that there was no significant difference between the buoyant density of hypoxic cells subjected to this kind of treatment and that of cells kept in a normal, oxygenated environment. This finding would therefore support the hypothesis that cells must be kept in a sustained hypoxic environment to effect a density change. On the other hand, tumor cells subjected to a "varying" or "cycling" type of oxygen tension in their environment may not develop a change in their buoyant density.

CHRONICALLY AND ACUTELY HYPOXIC TUMOR CELLS

The exciting aspect of all these studies would appear to be the suggestion that the hypoxic cells in tumors may be divided into at least 2 classes. The first class is the "chronically-hypoxic" cells described by Figure 1. The second class is the "acutely-hypoxic" cells which, unlike the chronically-hypoxic cells, do not remain hypoxic for extended periods of time. How would acutely-hypoxic cells develop in the tumor? One possibility would be that the vessels supplying oxygen and nutrients to the tumor tissues may be "opening" and "closing" for different periods of time as observed in many other vascular beds (Figure 4). The difference between chronic hypoxia and acute hypoxia is clearly understood if Figures 1 and 4 are compared. The usual explanation for the existence of hypoxic cells in tumors can only account for the chronic type of hypoxia. Regions immediately surrounding the blood vessel in Figure 1 never become hypoxic since they are situated well within the oxygen diffusion distance. In the situation proposed by Figure 4, even regions immediately surrounding the blood vessel can become transiently hypoxic if the blood flow to the vessel becomes interrupted. The buoyant density would suggest that in the EMT6 tumor, many if not all the hypoxic cells are of the acutely-hypoxic type.

Another question would appear to be relevant. Can acutely-hypoxic cells remain long enough to give the radiobiological response of hypoxic cells observed in the studies of Rockwell and Kallman (1973)? Experiments with mice tails performed by Wright and Howard-Flanders (1957) showed that if the blood supply to the tail was clamped off about 4 sec before irradiation a response of hypoxic cells

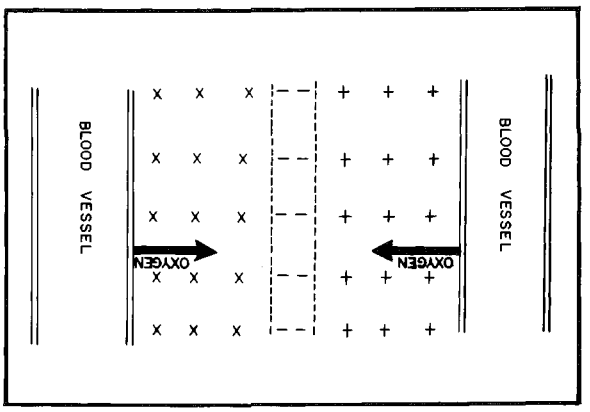

Fig. 4. Acutely-hypoxic cells in a tumor.

- - / - - Hypoxic/anoxic region situated about 150 μm from each blood vessel. This region represents the "chronically-hypoxic" region.

x x / x x Hypoxic region arising if the blood vessel on the right is shut (this region would represent the "oxygenated" cells if the blood vessel on the right remained open since it is closer than 150 μm away). If the blood vessel on the right remained shut, this region now represents the "acutely-hypoxic" region.

+ + / + + "Acutely-hypoxic" region if the blood vessel on the left remains shut.

was observed. Hence these data may suggest that tumor tissues need not remain hypoxic for extended periods of time before they would show a response typical of radiobiologically-hypoxic cells.

The studies of Thomlinson and Gray (1955) would most likely show only the regions which had been subjected to chronic hypoxia since acutely-hypoxic regions may not remain hypoxic long enough for them to die and necrose. Conversely, the presence of hypoxic cells in tumor as deduced from the "tails" of radiation survival curves probably detects both acute and chronic types. Therefore, it would seem that the presence of acutely-hypoxic cells can only be deduced through indirect experiments, as in the buoyant density experiments already described.

IMPLICATIONS FOR RADIOTHERAPY AND CHEMOTHERAPY

As previously stated, the ability to effectively sterilise hypoxic cancer cells may mean the difference between success or failure of radiotherapy or chemotherapy. A few years ago, some questions were raised regarding the clinical relevance of the oxygen effect since it was argued that doses currently used in radiotherapy should never have succeeded in curing any tumors if hypoxic cells are an important factor in *all* human tumors (Kaplan, 1974). It was pointed out that cells sustained in hypoxia for long periods may lose their clonogenic potential and thus be no longer of importance to radiotherapy. Nevertheless, the importance of hypoxic cells in determining the success of treatment of at least some human and rodent tumors is now quite well accepted (Hall, 1978). Thus it may well turn out that cells surviving an X-ray treatment are those of the acutely-hypoxic type, as their chronically-hypoxic counterparts may have lost their clonogenic potential. The phenomenon of reoxygenation described by Van Putten and Kallman (1968) may also involve the acutely-hypoxic cells. Thus the proportion of acutely-to chronically-hypoxic cells in a tumor may well be the factor determining the effectiveness of a treatment. Some drugs used in chemotherapy and other chemical modifiers used to modify the response of tumor cells to radiation (e.g. the radiosensitisers Metronidazole and Missonidazole) would apparently depend on their being able to diffuse into the poorly-vascularized and/or hypoxic regions of the tumor. In the situation of Figure 4, these chemicals may not be able to reach areas previously thought to be accessible by the situation of Figure 1 if the vessels remain closed for the period during which the drug/chemical is being delivered.

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REFERENCES

Hall, E.J. (1978). Radiobiology for the Radiologists, 2nd. Edition, Harper & Row Publishers, New York, Evanston, San Francisco and London.

Kaplan, H.S. (1974). On the relative importance of hypoxic cells for the radiotherapy of human tumors, *Eur.J. Cancer,* 10:275 280.

Moulder, J.E. and Rockwell, S.C. (1980). Survery of published data on the hypoxic fractions of solid rodent tumors, Radiation Research Meeting, New Orleans, Louisiana, 1980.

Ng, C.E. and Inch, W.R. (1978). Comparison of the densities of clonogenic cells from EMT6 fibrosarcoma monolayer cultures, multicell spheroids and solid tumors in Ficoll density gradients, *J.Nat. Cancer Inst.* 60:1017-1022.

Ng, C.E., Koch, C.J. and Inch, W.R. (1980a). Buoyant density of EMT6 fibrosarcoma cells : 1. Effect of hypoxic growth conditions *in vitro, Int.J.Rad. Biol. Oncol Phys.,* 6:481-486. .

Ng, C.E., Koch, C.J. and Inch, W.R. (1980b). Buoyant density of EMT6 fibrosarcoma cells: Time course of the density changes after growth in hypoxia, Cell Biophysics, 2:153-163.

Ng, C.E., Koch, C.J. and Inch, W.R. (1980c). Effect of ionizing radiation on buoyant density of EMT6 fibrosarcoma cells grown as monolayer cultures, multicell spheroids or solid tumors, submitted for publication.

Rockwell, S.C., and Kallman, R.F. and Felipe, F. (1972). Characteristics of a serially-transplanted mouse mammary tumor and its tissue culture-adapted derivative, *J. Nat. Cancer Inst.,* 49:735-749.

Rockwell, S. and Kallman, R.F. (1973). Cellular radiosensitivity and tumor radiation response in the EMT6 cell system, Radiat. Res., 53:281-294.

Thomlinson, R.H. and Gray, L.H. (1955). The histological structure of some human lung cancer and the possible implications for radiotherapy, *Brit.J. Cancer,* 9:539-549.

van Putten, L.M. and Kallman, R.F. (1968). Oxygenation status of a transplantable tumor during fractionated radiotherapy, *J.Nat. Cancer Inst.,* 40;441-451.

Wright, E.A. and Howard-Flanders, P. (1957). The influence of oxygen on the radiosensitivity of mammalian tissues, *Acta Radiol.,* 48:26-32.