Role of oxygenation and vascularization in drug resistance

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Received 25 May 1998; accepted 25 May 1998

Key words: drug resistance, oxygenation, tumors, vascularization

Abstract

Oxygenation status and tumor vascularization seem to be important factors in determining therapeutic effectiveness and patient prognosis. An abundance of data on tumor oxygenation and vascularization is available and it clearly shows that most human solid tumors are heterogeneously oxygenated and vascularized. They contain hypoxic regions. Such regions and areas of reduced vascularization can affect the response to a variety of drugs. Direct measurements of pO_2 and the vascular density in various types of tumors have, upon correlation of the data to therapeutic outcome, shown that low pO_2 values and low vascular density are associated with a decreased response to therapy. Therefore, oxygenation status and the extent of tumor vascularization may well be important factors contributing to the difficulty of successful therapy in certain types of tumors.

Introduction

Because of the development of new anticancer agents and improved chemotherapeutic regimens, cure rates for various types of human cancer have increased over the past decade. However, all these successes are limited by one of the major problems in cancer chemotherapy – drug resistance. A number of malignancies are relatively resistant to chemotherapy and radiotherapy. Several factors are involved in this lack of responsiveness. These include an intrinsic (genetically determined) resistance and an extrinsic resistance which is mainly caused by physiological and environmental factors such as inadequate and heterogeneous vascular networks (Vaupel *et al.*, 1989). Because the rate of neovascularization frequently fails to keep pace with tumor growth, tumor vasculature is often inadequate for the tumor mass. Thus, properties such as tumor blood flow, oxygen and nutrient supply, and the metabolic microenvironment can markedly influence the therapeutic response (Vaupel *et al.*, 1989). Alterations of the metabolic microenvironment and its accompanying therapeutic consequences are due to a number of structural and functional peculiarities shared by the newly constructed vasculature of malig-

nant tumors (Kallinowski, 1997). The proliferation of tumor cells is induced by activation of oncogenes or loss of suppressor genes while the structure and function of the tumor vessels is influenced by angiogenic factors. The complex network of possible interactions that involves genetic, structural and functional influences on the effectiveness of adjuvant therapies is simply described in Figure 1.

Many different types of human solid tumors are known to contain significant cell fractions that are hypoxic relative to the oxygenation of normal tissues. Evidence is increasing that hypoxia may be a significant contributor to tumor responsiveness in therapy (Kallinowski, 1996). Recently conducted research in angiogenesis shows that the extent to which tumors are oxygenated and vascularized is an important parameter for predicting the response to treatment (Gatenby *et al.*, 1988; Siracka *et al.*, 1988; Höckel *et al.*, 1993).

Methods for Measuring Tumor Oxygenation

A variety of techniques for measuring oxygen in normal and tumor tissues have been developed in the past (Raleigh *et al.*, 1996). These include: histo-

Figure 1. Influences on therapy-relevant parameters of the metabolic microenvironment of malignant tumors. These are subdivided into four hierarchic levels (according to Kallinowski, 1997). + = Stimulation, - = Inhibition.

morphometric analysis which measures the intercapillary distances in histological sections (Awwad *et al.*, 1986), using fluorescent dyes which specifically bind to hypoxic cells (Wardman *et al.*, 1984) and examining oxygen tension with microelectrodes (Kallinowski *et al.*, 1990; Höckel *et al.*, 1993) that can directly measure regional tissue pO_2 . This last method has been regarded as the most accurate and reliable in determining tumor oxygenation (Stone *et al.*, 1993). Introduction of the commercially available Eppendorf histograph system has facilitated intratumoral pO_2 measurements in human tumors and has permitted clinical investigations with larger patient cohorts to be conducted (Höckel *et al.*, 1996).

Methods for Measuring Tumor Vascularization

Presently, the method most frequently used to measure tumor angiogenesis in human solid tumors involves counting the intratumoral microvessels. This method requires using specific markers that react to the vascular endothelium and immunohistochemical procedures to visualize the microvessels. Intratumoral microvessels can be visualized with various endothelial specific antibodies such as factor VIII-related antigen (von Willebrand factor) and blood group antigens CD31

and CD34 (Weidner, 1995). Although CD31 (also known as the platelet/endothelial cell adhesion molecule PECAM) is apparently more sensitive, factor VIII-related antigen is thought to be the most specific endothelial marker (Weidner, 1993). Microvessel density in those tumor regions of greatest vascularization is usually measured with a light microscope. It has been demonstrated that choosing the most vascularized areas of a tumor (also called 'hot spots') for microvessel quantification yields information having the greatest prognostic validity (Weidner *et al.*, 1991). However, automated morphometric techniques, which provide the average microvessel count over a large tumor area, can make a comparable prognosis (Barbareschi *et al.*, 1995).

Alternative immunohistochemical methods for assessing angiogenic activity in cancers involve analyzing the intratumoral expression of angiogenic peptides that are responsible for vascular density in tumors. Such peptides may prove valuable as prognostic and therapeutic tools and include the vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF).

Table 1. Median pO₂ values in various normal tissues and patient tumors

Tissues	Median pO ₂ (mm Hg)
Spleen	66
Normal breast	65
Gastric mucosa	47
Skeletal muscle	28
Liver	24
Brain	24
Rectal cancer	25
Breast carcinoma	23
Head and neck cancer	19
Lung cancer	14
Cervical carcinoma	10
Melanoma	10

Adapted according to Vaupel *et al.*, 1989 and Vaupel and Höckel, 1995.

Oxygenation of Human Tumors

Using O₂-sensitive techniques employing microelectrodes, pO₂ distributions have been measured in various normal tissues and in human tumors. The data clearly show that most tumors are heterogeneously oxygenated and contain hypoxic regions. Variability in the oxygenation pattern from one tumor to another is often more pronounced than the heterogeneity within a single, specific tumor (Vaupel *et al.*, 1989; Lyng *et al.*, 1995). Relatively high pO₂ values are measured in regions of the tumor that are dominated by stroma and in the periphery. Low values are observed in tumor regions that have nearly necrotized as well as in more centralized regions. Comparing the O₂ concentrations of tumors to their normal tissue counterparts yields mean pO₂ values that are lower within malignancies, themselves, than in surrounding normal tissues (Vaupel, 1990) (Table I). In general, the oxygenation pattern in cancers and the occurrence of hypoxia do not correlate with stage, histology or tumor type.

The lower oxygen and nutrient levels have numerous effects on cell metabolism, the cell cycle and, ultimately, drug sensitivity (Teicher, 1994). Many studies indicate that hypoxic cells are significantly less sensitive to both radiation and chemotherapy than more well-oxygenated cells. A well-oxygenated cell is about 2–3 times more sensitive than its hypoxic counterpart (Moulder and Rockwell, 1987). During

the past few years, direct pO₂ measurements using microelectrodes and, afterwards, a correlation of such data to the therapeutic outcome have been undertaken (Kolstad, 1968; Gatenby *et al.*, 1988; Höckel *et al.*, 1996; Nordmark *et al.*, 1996). These studies demonstrate that the extent of tumor oxygenation serves as a prognostic factor indicating the effectiveness of radiotherapy in patients with advanced stage cervical cancer and carcinomas of the head and neck. Patients that had low pO₂ tumors and were treated with both radiotherapy and chemotherapy had a significantly shorter overall survival time as well as a reduced time interval free of recurrence than patients with high pO₂ tumors. Clinical studies focusing on various other tumor types are under evaluation.

Vascularization of Human Tumors

The oxygen status in tumors is determined by the vascularity, the blood flow, the oxyhemoglobin saturation and the oxygen consumption rate of the tumor cells (Vaupel *et al.*, 1989; Dewhirst, 1993; Teicher, 1995). Considerable variability in vascular density has been noted in different areas of one specific tumor, between different tumors having the same histology, as well as between tumors having different locations (Revesz *et al.*, 1989; Mattern and Volm, 1996) (Figure 2). While most tumor types exhibit a mean vascular density of <10 counts, renal and breast carcinomas exhibit significantly higher values than carcinomas located in other regions of the human body (Mattern and Volm, 1996). In general, vascular densities are lower in tumors than they are in normal tissues (Vaupel and Höckel, 1995).

Tumor vascularity appears to have a significant impact on the oxygenation status of many human tumors. Vascular density correlates well with the oxygenation status (Lyng *et al.*, 1996; Mattern *et al.*, 1996a). Well-vascularized tumors are better oxygenated and contain less hypoxic regions than poorly vascularized lesions.

Previous pathohistological studies of cervical cancer that associated a low vascular density in the tumors with a decreased response to radiation therapy indirectly provided evidence for a link between tumor oxygenation and radiocurability (Kolstad, 1968; Siracka *et al.*, 1988). A close correlation between therapeutic effectiveness and vascular density has also been described for head and neck tumors (Delides *et al.*, 1988) and breast carcinomas (Gasparini *et al.*, 1995).

Figure 2. Microvessel density in human tumors of varying localization (n = 152). Numbers in parenthesis represent the number of patients in the subgroups. The mean value of each group is shown by a vertical line.

Hypoxia and Up-regulated Genes and Proteins

During the past several years, some of the biochemical mechanisms that cause drug resistance have been identified. For example, resistance to a broad range of structurally different drugs is associated with an overexpression of the transmembrane transport protein, P-glycoprotein (Bradley *et al.*, 1988). Cell lines resistant to alkylating agents often demonstrate an enhanced expression of a group of cytosolic enzymes identified as the glutathione S-transferases (Wang *et al.*, 1989). Increased quantities of the target enzymes, dihydrofolate reductase or thymidylate synthase, have been shown in cells that are resistant to methotrexate or 5-fluorouracil (Schimke, 1984).

A growing body of evidence indicates that cells respond to hypoxic stress by altering the expression

of specific genes or proteins (Wilson and Sutherland, 1989; Sutherland *et al.*, 1996). Hypoxia-induced resistance to doxorubicin and to methotrexate has been attributed to an amplification of both the P-glycoprotein gene and the dihydrofolate reductase gene (Rice *et al.*, 1986; Rice *et al.*, 1987; Kalra *et al.*, 1993; Luk *et al.*, 1990).

However, this result was not confirmed in other studies (Wilson *et al.*, 1989; Sakata *et al.*, 1991). Murphy *et al.* (1994) have recently shown that metallothionein IIA mRNA levels were significantly increased during hypoxia and during reoxygenation. O'Dwyer *et al.* (1994) investigated the effects of hypoxia on the expression of a group of enzymes involved in drug metabolism. Exposing colon carcinoma cells to hypoxia resulted in a notably increased glutathione content, but did not yield an increased activity of

glutathione transferase.

Many of these changes have features in common with the response to radiation that induces an expression of several transcription factors (Angel and Karin, 1991; O'Dwyer *et al.*, 1994). For instance, c-jun protein is a component of the AP-1 transcription factor complex that is involved in the transcriptional regulation of several genes. It has been recently reported that the promoter region of the human MT gene contains the AP-1 binding site and that this site is essential for promoter activity (Lee *et al.*, 1987). The promoter region of genomic GST- also contains an AP-1 motif. This suggests that this gene may be regulated by the cellular oncogene c-jun (Morrow *et al.*, 1989). It has also been shown that hypoxic stress strongly induces expression of the c-jun proto-oncogene and, to a lesser degree, the related genes, c-fos and jun B (Ausserer *et al.*, 1994). Additionally, induction of the expression of certain detoxicating enzymes in human colon cancer cells upon exposure to hypoxia is caused by inducing both the transactivating factors that bind to the AP-1 element and the redox proteins that enhance their affinity for this element (Yao *et al.*, 1994).

It has been shown that poor vascularization in lung carcinomas, as measured by vascular density, correlates with an up-regulation of glutathione S-transferase, metallothionein and thymidylate synthase (Koomägi *et al.*, 1995). In another study involving rectal cancer, poor angiogenesis is also linked to an expression of glutathione S-transferase and metallothionein (Mattern *et al.*, 1996a). Furthermore, c-jun-positive and VEGF-positive tumors have significantly lower microvessel counts and pO₂ values than c-jun-negative and VEGF-negative tumors.

In a study of non-small cell lung carcinomas and renal cell carcinomas, it has been shown that, in resistant tumors, glutathione S-transferase and P-glycoprotein are overexpressed and that c-fos and c-jun proto-oncogenes might be involved in this process (Volm, 1993; Volm *et al.*, 1993). A remarkable parallel between biochemical changes in drug resistance and carcinogenesis has also been discovered. Lung carcinomas in smokers were more frequently resistant *in vitro*. They overexpressed glutathione S-transferase and P-glycoprotein and more frequently expressed c-jun and c-fos than did carcinomas in non-smokers (Volm *et al.*, 1991, 1992). These data suggest that an elevated detoxicating enzyme expression could be the consequence of hypoxic activation by immediate-early genes such as c-jun and c-fos. These, in turn, act on response elements in the promoter regions of

the genes that encode the detoxicating enzymes and other proteins such as VEGF. Therefore, the finding that hypoxia results in the overexpression of certain detoxicating enzymes provides an additional insight into hypoxic cell resistance (O'Dwyer *et al.*, 1994; Koomägi *et al.*, 1995; Mattern *et al.*, 1996a). Since different regions of tumors *in vivo* are variably hypoxic, this mechanism may contribute substantively to the constitutive resistance of tumors to drugs and radiation.

Hypoxia and Tumor Cell Proliferation

Hypoxia develops because of an inadequate vascular supply in relation to the mass of oxygen-consuming tumor cells. It has been shown that the commonly observed decreased tumor growth rate that is associated with an increased tumor volume might be due to vascular insufficiency in large tumors. Therefore, it is likely that cells that are located distal to the vasculature are poorly oxygenated, find themselves in an acidotic environment and are noncycling. Cells that are noncycling would be expected to be less sensitive to many agents.

Studies of tumor cell proliferation kinetics have indicated that the growth fraction decreases with increasing distance from the blood vessels (Tannock, 1968). It is well-known that tumors having a high growth fraction are more sensitive to therapy than tumors possessing a low growth fraction. The position in the cell cycle is also an important variable for the cytotoxic action of some chemotherapeutic agents. Some drugs are more cytotoxic towards proliferating cells or cells in S phase. Other drugs exert their maximal cytotoxic effect on non-proliferating cells (Valeriote and Van Putten, 1975). On the other hand, the effect of hypoxia on response to chemotherapy is also highly drug-dependent. Some drugs depend on oxygen for their cytotoxic effect. Other drugs are selectively cytotoxic towards hypoxic cells (Teicher *et al.*, 1981). Additionally, the environmental inadequacies that accompany hypoxia *in vivo* (e.g., low pH, nutrient deficiencies) can also inhibit proliferation (Wike-Hooley *et al.*, 1984).

Regulating the growth of new vasculature in tumors is important in determining the cellular responses that affect the clinical course. Among the various factors that regulate angiogenesis and affect tumor cell proliferation, VEGF and bFGF are known to be up-regulated by hypoxia (Shweiki *et al.*, 1992;

Kuwabara *et al.*, 1995). VEGF can act both as an autocrine growth factor in stimulating the proliferation of tumor cells (Mattern *et al.*, 1996b) and as a stimulator of endothelial cell proliferation (Connolly *et al.*, 1989). In a study of non-small cell lung carcinomas, it has been demonstrated that tumors with low expression of VEGF and low microvessel density were more frequently resistant to doxorubicin *in vitro* than were tumors with a high expression of VEGF and high vascular density (Volm *et al.*, 1996). These data indicate that not only reduced vascularization, but also reduced expression of VEGF may very well be important contributory factors to the high frequency of drug resistance. This, in turn, also contributes to the difficulty of successfully treating non-small cell lung carcinomas.

Therapeutic Implications

Angiogenesis is essential for progressive solid tumor growth. This fact constitutes a therapeutic target that holds great promise. Successfully inhibiting tumor angiogenesis may be a potential strategy for treating cancer. Presently, a number of strategies are employed in anti-angiogenic therapy (Bicknell and Harris, 1992; Folkman and Ingber, 1992). For example, strategies that may influence tumor growth include: inhibiting endothelial cell proliferation, the blocking activity of angiogenic peptides, inhibiting the formation of neovessels or stimulating inhibitors of angiogenesis. This antiangiogenic therapy should be distinguished from vascular targeting which is directly aimed at destroying the established vasculature by exploiting differences between the endothelia in normal and tumor tissues. Strategies that have been employed include antibodies that recognize tumorous, but not normal vasculature (Denekamp, 1993).

Hypoxic tumor cells, themselves, can be targeted through a number of different mechanisms (Rockwell, 1992). The targeting need not be mediated by hypoxia *per se*, but can instead be mediated through other microenvironmental perturbations that accompany hypoxia *in vivo*. For example, low pH, low glucose levels, high lactate levels or low levels of certain nutrients may provide a target for hypoxic cells. Many different agents have been shown to prefer toxic to hypoxic, acidic, or glucose-deficient cells *in vitro*. Clinical studies involving a variety of bioreductive agents and other hypoxia-directed drugs demonstrate that the application of hypoxia-directed cytotoxic drugs leads to the

selective killing of hypoxic cells. Such substances may prove useful as adjuncts to radiotherapy and chemotherapy in the treatment of solid malignancies (Rockwell, 1992; Kallinowski, 1996). In the future, methods employed in selecting high risk patients for adjuvant treatments will be important. Assessing the angiogenic and oxygenation status of individual tumors before therapy may be useful in selecting those patients who are more likely to benefit from adjuvant therapies.

Acknowledgement

We thank Dr. F. Kallinowski for critically reading the manuscript.

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