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Hypoxia-Driven Immunosuppression: A new reason to use thermal therapy in the treatment of cancer?

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Abstract

Hypoxia within the tumor microenvironment is correlated with poor treatment outcome after radiation and chemotherapy, and with decreased overall survival in cancer patients. Several molecular mechanisms by which hypoxia supports tumor growth and interferes with effective radiation and chemotherapies are now well established. However, several new lines of investigation are pointing to yet another ominous outcome of hypoxia in the tumor microenvironment: suppression of anti-tumor immune effector cells and enhancement of tumor escape from immune surveillance. This review summarizes this important information, and highlights mechanistic data by which hypoxia incapacitates several different types of immune effector cells, enhances the activity of immunosuppressive cells and provides new avenues which help “blind” immune cells to the presence of tumor cells. Finally, we discuss data which indicates that mild thermal therapy, through its physiologically-regulated ability to alter vascular perfusion and oxygen tensions within the tumor microenvironment, as well as its ability to enhance the function of some of the same immune effector activities that are inhibited by hypoxia,, could be used to rapidly and safely release the tight grip of hypoxia in the tumor microenvironment thereby reducing barriers to more effective immune-based therapies.

Keywords

hypoxia; anti-tumor immunity; thermoregulation; hyperthermia; tumor microenvironment

Introduction

Until recently, the underlying rationale guiding most cancer therapies was that the sensitivity of tumor cells to treatment arises from intrinsic characteristics of the tumor cells themselves and that these are expressed independent of the microenvironment. In other words, cancer cells within tumors have been treated to exploit potential vulnerable characteristics that are constitutively present, for example, increased tumor cell mitotic index which can aid in the efficacy of many chemotherapeutic drugs. Similarly, in the case of immunotherapies, a majority of studies have focused on the inherent immunogenicity (or lack thereof) of tumor cells. As a result, much work has been expended on efforts to strengthen weak tumor recognition by vaccinating patients with tumor antigens. Even in the much less studied field of thermal medicine, an area of intense interest to most readers of this review, one of the earliest and persistent rationales underlying the delivery of excess heat at the site of the

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tumor is focused on the ability of heat shock temperatures to enhance radiation damage through thermal modulation of the DNA repair pathway within individual tumor cells. As a result of this rationale, current hyperthermia treatment protocols aim at getting sufficient heat within tumors to engage these intrinsic radiation enhancing mechanisms.

However, current research indicates that the response of solid tumors to chemo-radiation therapy, immunotherapy, and perhaps even thermal therapy is not just a function of the inherent molecular properties of tumor cells or their ability to be recognized and killed by immune cells. Now there is an emerging appreciation of the role of the tumor microenvironment and of the normal cells that surround and infiltrate tumors in modulating the response of individual tumor cells to a variety of treatments and in altering the intrinsic phenotype of tumor cells. Important factors that influence the response of tumors to chemotherapy are the metabolic environment of the tumor cells and the basic vascular access of the drug to the tumor microenvironment. Defective vascular channels, combined with lack of homeostatic regulation seen in normal tissues, not only reduce drug uptake, but also contribute to the formation of hypoxic regions within tumors, a condition now known to promote growth of tumors and their ability to attract sufficient vascularization. Similarly in the case of radiation therapy, a lack of microenvironmental oxygen is the critical factor in preventing maximal DNA damage from radiation, limiting damage to a fraction of what would be possible if sufficient oxygen were available. In the case of immunotherapy, there is now a much greater appreciation for the fact that the degree of inflammation, and concentration of certain cytokines and chemokines within the tumor can significantly alter immunogenicity of tumor cells as well as immune cell activation potential.

This review covers new evidence indicating that the efficacy of the anti-tumor immune system may also be highly dependent upon the degree of hypoxia in the tumor microenvironment; thus hypoxia may not only blunt the effectiveness of chemotherapy and radiation, but may also contribute to an environment which inhibits the efficacy of natural host anti-tumor immune cells and improves the ability of tumors to avoid immunosurveillance. Further, this review will highlight some reasons why the use of thermal therapy, (particularly mild thermal therapy) through its ability to activate naturally occurring thermoregulatory homeostatic processes (which in turn could modulate tumor vascular perfusion and reduce regions of hypoxia), as well as its ability to stimulate immune cell activity, could enhance the natural immune response against cancer. We speculate that reversal of hypoxia-induced tumor immunosuppression could, at least in part, help to explain the positive survival benefit and reduction in local tumor recurrence seen following the use of hyperthermia in combination with other cancer therapies in multiple Phase II and Phase III trials. Specifically, over the past 15 years, there has been an exciting accumulation of data indicating a positive survival benefit when hyperthermia is added to radiotherapy and/or chemotherapy, validating early literature suggesting just such a benefit (1). There have been positive phase III trials for cancers including: melanoma (2), esophageal cancer (3,4), locally advanced head and neck cancer (5), locally advanced cervix cancer (6) and gliomas (7). Addition of hyperthermia contributes to superior local control and durable responses in chest wall recurrences of breast cancer, when combined with radiotherapy, as compared with radiotherapy alone (8,9). These successes are driving a resurgence of interest in understanding the potential mechanisms by which temperature affects the efficacy of cancer therapy.

This exciting clinical trial data support the value of renewed, vigorous research efforts to test this and other new hypotheses regarding mechanisms by which thermal therapy improves cancer patient survival, and to develop and test new heating strategies which may maximize immunological and physiological anti-tumor effects.

The importance of hypoxia in tumor biology and patient prognosis

For decades, cancer researchers have been aware of the fact that there are regions of mild to severe oxygen deprivation in solid tumors (10) that are most likely due to aberrant vascular function as well as metabolic abnormalities associated with rapid tumor growth (11). Clinical investigations have reported that a large fraction of locally advanced solid tumors demonstrate a prevalence of hypoxic tissue (12). Determining the extent of hypoxia within patient tumors has been achieved using techniques such as microelectrode probes, nitroimidazole based compounds, PET imaging, and biomarker expression by immunohistochemistry (13-15). Solid tumors with measured oxygen tension levels less than ~ 2.5 to 10 mm Hg are considered hypoxic and are positively correlated to enhanced tumor progression and increased therapeutic resistance (16). In a cervical cancer study published in 1993, it was found that the extent of hypoxia positively correlated with a more negative prognosis (17,18). In recent years, hypoxia inducible factor (HIF) −1α expression in both pancreatic cancer and surrounding stromal cells has been correlated to poor patient survival (19). Tumor hypoxia and patient outcome have been further studied in other malignancies such as head and neck (20), prostate (21), and breast (22,23) and in each case, hypoxia was seen to predict a more negative clinical outcome. In a study on patients with soft tissue sarcoma, Brizel et al showed that tumor oxygenation status predicts for the likelihood of distant metastases (24).

While it may seem intuitive that hypoxic conditions could slow or even block the growth of a tumor, unfortunately tumor tissues are able to respond to this stress in a manner which protects and supports their growth. Indeed, it has been reported that certain clones within the tumor can react favorably to hypoxic conditions, leading to tumor progression (25,26). Moreover, a state of hypoxia promotes the development of new blood vessels for supply of nutrients and oxygen (27). Hypoxic conditions have been reported to affect gene expression associated with increased angiogenesis, resulting in an increase in VEGF and receptor levels (28). These angiogenic signals induced by hypoxic conditions permit the continued growth and survival of the tumor cells leading to a potentially progressive and invasive phenotype.

How does malignant tissue become hypoxic? There have been numerous investigations into this important question and several outstanding reviews of the literature have been published. While the amount of oxygen carried by the blood to *normal* organs and tissues is more than sufficient to meet their metabolic requirements, the consumption rate of oxygen in neoplastic and stromal cells in locally advanced tumors appears to be greater than the supply, resulting in areas of low O_2 levels (22). Possible pathogenic mechanisms involved in development of tumor hypoxia according to one analysis of the literature by Vaupel (29) are 1) perfusion-limited O_2 delivery, 2) diffusion-limited O_2 delivery, and 3) anemic hypoxia. Perfusion-limited O_2 delivery is the result of aberrations and functional changes in the tumor microvessels, resulting in limited O_2 into the tissue (30). Diffusion-limited delivery is caused by cells that are located too far away from nutrients and oxygen supplied from blood vessels, leading to enhanced hypoxia in that tissue area (31).

In a more recent review of the literature, Dewhirst et al. (11) identified some similar and a few additional causes of tumor hypoxia. These include 1) the relatively sparse arteriole supply in many tumors, 2) an inefficient orientation of tumor blood vessels leading to an overabundance of vasculature in some regions and insufficient vasculature in others, 3) large variations in flow velocity and in the number of red blood cells that traverse a microvessel per unit time, 4) effects of hypoxia on red blood cells, which have been reported to shrink and become more stiff than normoxic cells, and increases blood viscosity slowing flow, 5) the existence of large diameter shunts between arteriolar and draining veins diverting blood and oxygen away from the tumor mass, 6) increased metabolic demand for oxygen in

tumors, in addition to the observation that the binding of oxygen or heightened metabolism of tumor cells nearest the microvessels may limit penetration to deeper layers. Moreover, Minchinton and Tannock (32) observe that the packing density and cell to cell adhesion between tumor cells may limit penetration of oxygen to deeper cell layers. These authors also highlight the importance of intra-tumoral pressures in helping to compress tumor blood vessels, interfering with effective delivery of blood (33). All of these mechanisms contribute to the occurrence of hypoxic areas in tissues which can lead to enhanced tumor progression and increased malignancy. Subpopulations of tumor cells that survive and progress under the nutrient deprived conditions are hypothesized to produce more aggressive, therapeutically resistant disease (34). For example, in one recent study, evidence was provided that prostate cancer cells exposed to chronic hypoxia caused these tumor cells to have a more aggressive phenotype and display a higher invasion activity in matrigel assays than cells cultured under normal conditions (25).

Following acute oxygen deprivation in normal cells, a key regulatory protein HIF-1α functions to maintain homeostasis. HIF-1α becomes stabilized, heterodimerizes, and translocates to the nucleus and binds to hypoxia-responsive elements (HRE) of several genes responsible for increasing oxygen and nutrients within the hypoxic tissue. However, under conditions of chronic hypoxia within a tumor microenvironment, constitutive HIF-1α expression can occur and aid in tumor progression, invasion and metastasis. HIF-1α expression in tumors has been reported to induce epithelial to mesenchymal transition (EMT) in colon cancer, increase expression of angiongenic factors such as VEGF, and increased genetic instability (35,36). These factors can lead to hypoxia-mediated resistance to apoptosis, decreased DNA repair and increased mutagenesis rates.

Tumor hypoxia results in enhanced resistance to radiation and chemotherapy

Tumor blood flow and related microenvironmental parameters (tumor tissue oxygenation, pH, nutrition supply, and interstitial fluid pressure) are believed to significantly impair cancer therapies (37-43). It has been previously shown that radiosensitivity significantly decreases when pO_2 pressure is less than 30 mmHg in the tumor (22). A two or three fold higher radiation dose is needed to kill hypoxic tumor cells compared to well-oxygenated cells (14,44,45). Many clinical studies have reported pO2 of less than 15 mmHg in different types of tumors and therefore it is clear that an improved ability to predict the extent of hypoxia (and modify it) *before or during* radiation therapy could provide invaluable clues for designing alternative treatment schemes.

Increased HIF-1α levels both experimentally and clinically have been shown to be correlated with a decrease in tumor radiosensitivity. For example, in a recent study, it was shown that lung cancer cells express high levels of HIF-1α and are resistant to radiation induced cell death (46,47). Thus, HIF-1α is a promising target for increasing radiosensitivity of tumors that display a hypoxic phenotype. Using inhibitors to HIF-1α or proteins involved in its regulation could provide a way to increase the sensitivity of tumors to radiation therapy (48,49).

Many cytotoxic drugs are also dependent on oxygen and tumor hypoxia could confer resistance to chemotherapeutic drugs. Constitutive activation of HIF-1α and subsequent activation of many HRE genes such as STAT3 leads to chemoresistance. Constitutive activation of STAT3 in ovarian cancer cells cultured under hypoxic conditions renders them resistant to chemotherapeutic drugs such as Cisplatin and Taxol (50). A recent study by Huang et al. observed that hypoxia induced HIF-1α expression in ovarian cancer cells decreased the cells susceptibility to Paclitaxel (51). Another study reported that hypoxia can

also reduce p53 protein levels in tumor cells and results in resistance to Etoposide therapy (52). It is clearly evident that hypoxic conditions can confer resistance of tumor cells to chemotherapies and hypoxic-induced proteins such as HIF-1α need to be targeted to increase efficacy of these chemotherapies.

The above data summarizes some of the literature which indicates the importance of hypoxia in blunting more effective radiation and chemotherapy. The immune system is also a potentially powerful force that can prevent or slow tumor growth and major efforts are underway to develop new therapies that exploit anti-tumor immune responses. Unfortunately, there is now growing evidence that hypoxia can also negatively impact immune cell function and limit effective immunosurveillance. In the following section, we summarize some of the information on this important field of research.

Immunosuppressive effects of the hypoxic tumor microenvironment

Accumulating evidence suggests that a hypoxic microenvironment may protect tumors from natural anti-tumor immune responses (and from immunotherapies) by inhibiting anti-tumor immune effector cells and facilitating immune escape. Examples that will be described more completely below include 1) tumor hypoxia induced tumor cell shedding of immune recognition molecules, reducing sensitivity to NK or CTL-mediated killing, 2) hypoxia-induced inhibition of dendritic cells and T cells, and 3) hypoxia-induced promotion of suppressive cells (T regulatory cells and tumor-associated macrophages) which in turn, block immune effector cells (see Table 1 for a summary of key points from this literature). However, as will also be indicated below, several of these same immune effector cells may be positively affected by mild thermal stress, thus providing a potential rationale for using thermal therapy in cancer treatment.

Hypoxia-induced shedding of immune recognition cell surface markers

NKG2D is an activating receptor expressed by natural killer (NK) cells, $CD8^+$ T cells and γδ T cells. The binding of NKG2D to its ligands activates NK and T cells and promotes cytotoxic lysis of the cells expressing these molecules. The MHC class I chain-related (MIC) molecules (MICA, MICB, and UL16-binding proteins), one family of the NKG2D ligands, are not expressed by the majority of benign cells, but are upregulated on numerous tumor cells (53,54). The MIC molecules expressed on tumor cells are important for tumor immune surveillance through their interaction with NKG2D receptors on NK and cytotoxic T cells and subsequent tumor lysis (55-57). Several lines of evidence show that shedding of MIC molecules is one of the mechanisms used by tumor cells to escape from immune surveillance. Tumor-derived soluble MIC ligands (sMIC) down-regulate and degrade NKG2D on T cells and impair these tumor-antigen-specific effector T cells (58). Serum levels of sMIC from colorectal cancer patients are elevated and mediate down-regulation of activating NKG2D receptors on NK cells (59).

Studies on prostate cancer cells have shown that hypoxia *increases* tumor cell shedding of MIC molecules through impaired nitric oxide (NO) signaling. This hypoxia-induced MIC shedding decreases the sensitivity of tumor cells to peripheral blood lymphocyte-mediated killing. This finding is important because previous studies have shown that hypoxia-induced tumor invasiveness and chemoresistance are linked to reduced nitric oxide (NO) signaling (60,61). NO mimetic treatment attenuates hypoxia-induced shedding of MIC molecules and decreases prostate tumor growth in a murine xenograft model (62). These results suggest that reactivation of NO signaling through administration of NO mimetic agents to increase cancer cell MIC expression can be a potential immunotherapy and help to overcome hypoxia-driven tumor escape.

Impaired dendritic cell maturation and cytokine production within hypoxic tumor microenvironments

Dendritic cells (DC) and tumor antigen-specific T cells are essential for optimal anti-tumor immunity. Immature dendritic cells have acute ability to capture antigens, including tumor antigens but low capacity to stimulate T cell activation. Upon antigen uptake, DC can undergo maturation and express high levels of MHC, CD40, CD80 and CD86. DCs will then migrate to lymph nodes during their maturation process and present antigen to T cells for activation and initiate adaptive immune responses (63-65). Factors which could inhibit DC maturation or function in the tumor microenvironment might be a very important mechanism for tumor immune escape. Triozzi et al. generated autologous DCs *in vitro* from peripheral blood of melanoma and breast carcinoma patients. Intratumoral injection of these *in vitro* generated DCs induced tumor regression in some patients along with lymphocyte infiltration and tumor necrosis. However, these DCs failed to induce a systemic response because they apparently could not migrate normally out of the tumor tissue to draining lymph nodes (66). Recently, Yang et al. used human monocyte-derived DC and cultured these cells under normoxic or hypoxic (1% O_2) condition for 5 days in the presence of GM-CSF, IL-4. Maturation was further induced by LPS treatment. They found that hypoxia could inhibit DC maturation and cytokine production by inhibiting CD40 and MHC expression, as well as, Th1-type cytokine production (eg, IFN-γ, TNF-α and IL-12). These hypoxia-modified DCs displayed poor T cell stimulatory activity and were skewed to a Th2-stimulating phenotype by polarizing naïve T cells to secrete IL-4 instead of IFN-γ (67). Th1 cells can enhance the function of tumor-specific CTL through co-stimulatory molecules present on their surface and also indirectly by secretion of IL-2 (68). Therefore, hypoxia-skewed Th2 cell development will compromise anti-tumor immunity (69). Overcoming hypoxia within tumors would therefore seem to be necessary for achieving effective dendritic cell function leading to a Th1 response.

Hypoxia reduces T cell proliferation and survival and inhibits CTL development

Activation of tumor antigen-specific T cells is a critical event needed for anti-tumor immune surveillance. T cells are exposed to different oxygen tensions, including hypoxic levels, during their development and during migration between blood and tissue (70). A hypoxic environment can exist normally in lymphoid organs, inflamed tissues and tumors. T cells have been shown to increase expression of certain genes which are regulated by HIF (VEGF, glycolytic enzymes) after exposure to hypoxia (71-73). Many studies report that *hypoxia has inhibitory effects on T cell growth.* For example, T cell growth and survival are impaired at low oxygen levels because hypoxia will downregulate T cell IL-2 mRNA expression (74).

Lymphocytes express both voltage-dependent potassium (Kv) and Ca^{2+}-activated potassium (Kca) channels and their activities are essential for T cell activation (75). K^+ channels modulate the resting potential of the T cell membrane and indirectly regulate Ca^{2+} signaling which is important for cell proliferation and cytokine production. It is well established that blocking Kv channels inhibits T cell proliferation by inducing membrane depolarization and decreasing calcium influx (76). Conforti et al. have shown that hypoxia selectively inhibits TCR-mediated T cell proliferation by downregulating the expression, as well as activity, of Kv channels. On the other hand, T cell proliferation induced by agents bypassing the membrane (such as ionomycin with or without PMA) is not affected by hypoxia. Therefore, the Kv channels expressed in T cells are sensitive to hypoxia and are responsible for hypoxia-mediated inhibition of T cell proliferation (77).

Differentiation of T cells is both TCR-driven and cytokine-dependent. Therefore, it is very important to evaluate the effect of hypoxia on T cell cytokine production. Caldwell et al

have shown decreased $CD8^+$ T cells development under hypoxic conditions (2.5% O_2) compared to normal oxygen concentration (20% O_2). Hypoxia also alters CTL cytokine secretion patterns. Hypoxia exposure enhances the transcription of HRE-containing genes, such as VEGF but inhibits the accumulation of non-HRE-containing genes, such as IL-2 and IFN-γ during TCR-driven activation. This suggests that T cell activation under hypoxic condition *in vivo* may lead to different patterns of cytokine secretion (78).

Since reduction of IL-2 production is the major consequence of hypoxia-induced T cell immunosuppression, Kim et al. engineered human tumor-specific cytotoxic T cells which express hypoxia-inducible human IL-2 genes (HRE-IL-2) to rescue CTL function within a hypoxic tumor microenvironment. They found that these modified CTLs sustained their proliferation and survival by increasing IL-2 production under hypoxic conditions. HRE-IL-2 transduction also increased CTL cytotoxic activity even under hypoxia. After adoptive transfer into tumor-bearing mice, these HRE-IL-2-modified CTLs migrated into the tumor and promoted more rapid and complete tumor regression than parental CTLs. Overall survival was also increased after the HRE-IL-2-modified CTL transfer (79). Therefore, increase of T cell growth and survival within tumor hypoxic microenvironments, by providing IL-2, can restore their anti-tumor functions and may overcome hypoxia-induced immunosuppression.

Hypoxia- and adenosine receptor-mediated T regulatory cell development and suppressive function

Cells which are now known to help suppress effective anti-tumor immunity include T regulatory cells (Tregs) and tumor-associated macrophages (TAMs). Treg cells play an important role in suppressing immune responses for central or peripheral tolerance but they also protect cancer cells from anti-tumor immunity. Treg cells exert their suppressive effects by direct contact with T effector cells, inhibition of DC-induced T cell priming and secretion of suppressive molecules (TGF-β, IL-10, galectin-1, and CTLA-4) (80). A more complete understanding of the development and activities of Treg cells may help generate new therapies to inhibit their tumor growth promoting functions.

A very intriguing hypothesis regarding the effects of hypoxia-induced immunosuppression is now emerging: the tumor hypoxic microenvironment not only actively inhibits anti-tumor immune cells, but also promotes development of immune suppressor cells and this effect involves a role for adenosine in the tumor microenvironment. It has been shown that one consequence of local hypoxia is the accumulation of extracellular adenosine. Specifically, hypoxia upregulates adenine nucleotide-metabolizing ectoenzymes, ATPase/ADPase, CD39 and 5′-nucleotidase, CD73 to increase extracellular adenosine production (81). The extracellular adenosine signals through high-affinity A_{2A} adenosine receptors on activated immune cells and increases immunosuppressive intracellular cAMP. Signals triggered from A_{2A} adenosine receptors result in an “off” signal to inhibit immune cell activation, such as inhibiting the release of tissue-damaging oxygen radicals in polymorphonuclear neutrophils, downregulating APC cytokine production and antigen presentation, and inhibiting peripheral T cell proliferation and activation. This mechanism may help to down-regulate immune responses to protect normal tissues from collateral inflammation-induced tissue damage (82). However, the potential for hypoxia-adenosinergic signaling may be underestimated because the majority of current *in vitro* studies are at non-physiologically high (~21%) oxygen concentration (78).

Natural Treg cells express high levels of CD39 and CD73, which are responsible for extracellular adenosine production. Treg cells also have high intracellular cAMP and produce extracellular adenosine themselves. Therefore, based on the immunosuppressive cytokine upregulation in other cell types under hypoxic conditions, Sitkovsky et al. (83)

have proposed a model for Treg cell development and suppressive function via hypoxia-driven and adenosine receptor-mediated (hypoxia-adenosinergic signaling) within the hypoxic tumor microenvironment. They propose that TCR-activated Treg cells which express cAMP-elevating A_{2A} and HIF-1α might lead to enhanced transcription of immunosuppressive molecules such as TGF-β, IL-10 and galectin-1 which have hypoxia response element (HRE) and cAMP response element. In addition, A_{2A} and HIF-1α upregulate CD39 and CD73 on Treg cells to mediate extracellular adenosine accumulation to directly inhibit DC and T effector cell functions. Hypoxia also increases FoxP3 expression in a HIF-1α-dependent manner (84,85). Therefore, the A_{2A} and HIF-1α pathways might be required for Treg cell development (83). These new data suggest that tumor hypoxia is beneficial for Treg cell development and function, thus leading to significant anti-tumor immune suppression.

Tumor-associated macrophages: a pro-tumor phenotype activated by tumor hypoxia

Phagocytic cells such as neutrophils and macrophages are important cells of the innate immune system and they function in response to tissue injuries or invading pathogens during inflammation. One feature of inflamed tissue is hypoxia and therefore, the function of phagocytic cells could be modulated by altered concentrations of oxygen or other aspects of abnormal metabolic activity. Phagocytes may need to adapt to these hypoxic environment to generate energy and function efficiently (86). Compared to other immune cells, phagocytes switch their metabolic activity to use anaerobic glycolysis to generate ATP (87-90). Deletion of HIF-1α in phagocytes revealed that HIF controls major defense functions in phagocytes including phagocytosis, production of bactericidal molecules and pro-inflammatory cytokine production. Under hypoxic conditions within inflamed tissues, HIF can synergize with the NF-κB pathway to increase phagocytosis of bacteria, release of bactericidal molecules and pro-inflammatory cytokines and inhibit apoptosis to increase phagocyte lifespan (91).

Cancer cells can secrete many chemoattractants to recruit monocytes into tumors (92). Recruited monocytes rapidly differentiate into immunosuppressive tumor-associated macrophages (TAMs) and accumulate in the hypoxic area. In comparison to conventional macrophages, TAMs have a relatively immature macrophage phenotype and have poor antigen-presenting ability and produce factors that suppress T cell proliferation and activity. TAMs also up-regulate several genes (e.g., growth factors, VEGF, MMP-7) to promote tumor growth, invasion and metastasis. New data suggests that hypoxia appears to entrap TAMs by decreasing their mobility (91,93,94). These results suggest that hypoxia can recruit blood monocytes and activate a pro-tumor phenotype in macrophages to promote tumor growth.

To conclude this discussion, the immune system normally up-regulates many genes to adapt to hypoxic conditions in order to maintain their functions in various regions of the body that may differ in terms of oxygen tensions. However, as outlined above, cancer cells take advantage of hypoxia to alter activities of several immune effector mechanisms and increase tumor cell escape from immune surveillance. A better understanding of the mechanisms of hypoxia-induced immunosuppression is needed to develop more efficient therapies to boost anti-tumor immune responses. Moreover, these data contribute to the need for testing new ideas for reducing hypoxia within tumors.

Mild, fever-range hyperthermia positively affects several of the same immune mechanisms negatively affected by hypoxia

Previous studies from our lab (95-99) have shown that fever-range thermal stress can modulate some of the same immune targets that are affected by hypoxia and fortunately,

these data suggest the effects of mild thermal stress are opposite to that of hypoxia. For example, we observed that the cytotoxic activity of human NK cells isolated from peripheral blood is enhanced after exposure to fever-range thermal stress and this correlates with an increase in NKG2D clustering but not total level of NKG2D surface expression. Unlike hypoxia, mild temperature elevation (i.e. 39.5°C) also results in the up-regulation of MICA on tumor target cells that is associated with increased sensitivity to cytolysis in our in vitro studies. These results suggest that fever-range thermal stress not only enhances tumor cell recognition through upregulation of molecules on tumors needed for NK cell recognition, but also increases NK cell cytotoxicity (95,99).

Dendritic cell mobility is also sensitive to temperature changes. Unlike hypoxia, fever-range thermal stress enhances DC activation by increasing MHC II and CD86 expression. Our in vivo studies show that DCs exposed to mild fever temperatures have increased mobility which is likely associated with increased DC trafficking to the draining lymph nodes. Besides DC phenotype and migration patterns, hyperthermia allows for greater T cell activation in a mixed lymphocyte assay (96-98,100). Therefore, opposite to the effects of hypoxia, mild thermal stress enhances DC activation and antigen presentation which may lead to an increased anti-tumor T cell response. In our new in vitro studies, we have observed that fever-range thermal stress directly increases CD4 T cell IL-2 production in response to a suboptimal activation stimulus. IL-2 is a critical cytokine needed to increase and mobilize immune effector cells, and further work indicates that its enhanced production may be related to thermally-induced changes in T cell membrane lipid raft organization (M.Yuan and M.Grimm et al., unpublished MSS). Overall, these results suggest that thermal therapy could be used to help reverse at least some of the negative effects of hypoxia on the anti-tumor immune response. However, to date, there are few studies that evaluate the effects of mild thermal stress on some of the other immune suppressive mechanisms (e.g., recruitment of Tregs or TAMs) discussed above and therefore this should be a top research priority.

Thus mild thermal therapy may have a dual benefit: direct enhancement of immune cell activity through thermally sensitive molecular pathways associated with immune cell function/activation, and, indirect enhancement of immunosurveillance through a reduction in hypoxia-induced immune suppression via improved tumor vascular perfusion. In the next section, we discuss the possibility that mild thermal therapy can also be used to relieve, at least temporarily, the grip of hypoxia in the tumor microenviroment through its effects on vascular function.

Hyperthermia-induced effects on vascular perfusion of tumors: Can thermal therapy help to overcome hypoxia-driven immunosuppression in the tumor microenvironment?

Several lines of research suggest that tumor vasculature may be an important target of hyperthermia. Indeed, a well studied aspect of fundamental vertebrate physiology is the exquisite neuromuscular-mediated vascular homeostatic mechanisms *in normal tissue* that are launched rapidly and reversibly to change vascular flow patterns whenever there is even a very small change in tissue temperature. Extensive literature has revealed the fact that heat-induced changes in blood flow (from exercise, fever, or changes in ambient temperature) is due to controlled amounts of both vasoconstriction and vasodilation of blood vessels which are well supplied by sympathetic nerves, which in turn are regulated through the action of the temperature-regulating centers of the hypothalamus, as well as exquisitely sensitive warm and cold receptors in the skin and in deep tissues (101-105). But, does this same high degree of neuronal-vascular thermoregulation occur in the tumor

microenvironment? Currently, little evidence is available to answer this question. For example, it is not known whether tumor blood vessels differ from normal vessels in terms of the density of warm/cold receptor nerve endings. Cancer researchers believe that the blood vessels which supply tumors come from nearby normal host vessels which become incorporated into growing tumors and/or *from newly formed vessels.* Whatever their source, tumor vasculature often exhibits severe functional and morphological abnormalities; sympathetic nerve endings are missing on newly formed blood vessels, and these vessels are often seen to be highly irregular in their course, with dilated and compressed regions, irregular branching, and poor perfusion (106). Incomplete or missing endothelial cells and smooth muscle cells, interrupted basement membranes may result in increased vascular permeability, contributing to an increased interstitial fluid pressure (IFP) (32). The lack of normal innervation and the existence of numerous structural defects, including deficient smooth muscle coating cells would strongly predict that the tumor microenvironment may actually escape the active neuro-regulatory control which exists in the rest of the body following heating. But, curiously, within the tremendous volume of literature on neurovascular thermoregulatory mechanisms, there is no comparison of normal and tumor blood vessels, nor an examination of whether tumors differ from normal tissue in terms of the density of thermal nerve endings or in terms of their ability to actively constrict or dilate in response to temperature shifts. Nevertheless, early studies in the field of hyperthermic oncology have looked at blood flow in tumors following local heating and this information is briefly summarized here. Although the data are complex (with some differing conclusions that may depend upon the local heating protocol/duration, temperature achieved or tumor model used) a series of studies have shown that hyperthermia can change tumor oxygen concentration, blood flow and vascular permeability, and these factors could contribute significantly to overcome hypoxia induced immunosuppression. Below is a brief summary of some of this pioneering literature on tumor oxygenation and blood flow in response to local hyperthermia, followed by some new information from our own studies using systemic heating at mild, fever-range temperatures.

Hyperthermia-induced changes in tumor oxygenation and vascular perfusion

Hyperthermia has been suggested to increase the responses of tumor cells to radiation by several mechanisms, including improving tumor oxygenation (107,108). See Table 2 for a brief summary of some of these studies on tumor oxygenation measurements. This idea has been supported by data as far back as the 1980s. Bicher et al. (109), Tanaka et al. (110) and Vaupel et al. (111) had reported that increased tumor oxygenation occurred after heating at mild temperature in rodent tumors. Several more recent studies in rodent tumors, human xenografts, patient and canine tumors support that local heating (40-43°C) results in an overall improvement of tumor oxygenation. The changes in tumor oxygenation after heating correlated with changes of tumor blood flow (109,112-115). The increase of oxygen delivery into the tumor through increased blood flow after heating could reduce hypoxic regions within the tumor microenvironment. A higher thermal dose (>43°C) leads to decrease of tumor oxygenation possibly because of blood vessel damage (40,108,114,116-118).

In these studies, heat-induced tumor oxygenation is transient, persisting for several hours and then begins to decrease depending on the heat dose and tumor type (119). Other studies also show that heating tumors grown at different sites (in the hind limb, leg or flank) may result in different changes in tumor pO_2 (110). Collectively, these important studies show that the effects of hyperthermia on tumor oxygenation depend not only on the heat dose but also on the tumor site. Moreover, it has been suggested that the late increase in tumor pO_2 (24 hour after heating) is not as proportional as that found immediately after heating. This

suggests that the late increase in tumor pO_2 might also be controlled by other mechanisms such as a decrease in oxygen consumption (120,121).

In addition to studies on tumor oxygen levels, there are also many studies on tumor blood flow after local hyperthermia which reveals complex results. The changes in blood flow caused by hyperthermia seem to be dependent on heating temperatures, lengths and tumor types. Heating at lower temperatures (41-43.5°C) significantly increases the blood flow in SCK tumors in A/J mice (122), RIF-1 tumors in C3H mice (123), and spontaneous canine tumors (124). Similar results have also been found in human xenograft tumors (125). However, heating at higher temperatures (44.5°C) will decrease the blood flow (64,122-124) and this may be due to direct damage to blood vessels. The responses of multiple heating on tumor vessels are quite different from that of single heating. In R3230 AC tumors, the tumor blood flow after heating at 42.5°C for 1 hr is less than in the control tumors due to vascular damage. However, when tumor are preheated at 42.5°C for 1 hr and reheated 16-24 hr later at 42.5°C, the tumor blood flow increases two to three times after the second heating. The increase in tumor blood flow by the second heating following the conditioning of blood vessels by the first heating has been suggested to be due to development of vascular tolerance. (126).

All of the above studies have utilized local or local/regional heating models in rodents and used relatively high, non-physiological temperatures (40°C and above) to mimic clinical protocols in which heat shock temperatures are used. Because thermoregulation is optimally regulated at physiologically relevant temperatures (below 40°C), we have concentrated our studies on temperatures from 38 to 39.5°C and have developed a systemic heating model rather than trying to use a local heating model. Using this model, earlier studies from our lab showed that *systemic* mild (fever-range) hyperthermia results in an obvious expansion in the diameters of many tumor blood vessels and an increase in the percentage of perfused blood vessels with discernable erythrocytes (99,100). This thermally-increased perfusion can persist for 24-48 hours after heating (127). As mentioned earlier, high tumor interstitial fluid pressure may contribute to insufficient blood perfusion and reduced oxygenation and is recognized as the barriers for tumor therapy. New data from our lab shows that *systemic* or whole body hyperthermia treatment can significantly decrease tumor interstitial fluid pressure (IFP) to a comparable level achieved in Taxol treatment, a common chemotherapy drug that has been shown to decrease tumor IFP. (Arindam Sen et al, unpublished MSS) Systemic heating may indeed trigger powerful thermoregulatory responses which could in turn, be responsible for increased vascular perfusion within tumors and reduced tumor IFP.

While there are still many unanswered questions, collectively, the data in this field suggest that hyperthermia, (both local applications at higher target temperatures and systemic application at physiologically relevant temperatures) increases tumor oxygenation and vascular perfusion. The expected resultant decrease in hypoxia could provide a tumor specific window of opportunity for decreasing the immunosuppressive environment within the hypoxic tumor microenvironment. Moreover, direct immune activation by thermal signaling can further enhance anti-tumor immune effector activity. (See the article by Multhoff in this Special Issue for a more in depth study on immune cell activation/heat shock protein function and hyperthermia.) More research is needed to compare various temperatures and protocols for achieving tumor hyperthermia and to more completely test these exciting assumptions.

Summary and identification of Additional important Research Questions

While hypoxia is well known to contribute to radio- or chemoresistance of tumors, this article summarizes a growing body of evidence showing that the hypoxia within the tumor

microenvironment can also be highly detrimental to the activity of the anti-tumor effector mechanisms. Specifically, we summarize data which shows that hypoxia not only helps tumor cells to escape recognition of cytolytic cells (e.g., by inducing shedding of targets for NK cells) but also prevents the proper maturation and function of dendritic cells and T lymphocytes and increases the potential for recruitment of immunosuppressive T lymphocytes and tumor associated macrophages. Hypoxia induced immunosuppression can be added to other known strategies by which tumors escape effective immune control either by natural immunity or following immunotherapy.

Although the mechanism is far from clear, local hyperthermia can increase oxygenation of tumors and increase vascular perfusion in experimental animal tumor models. Whether the effect of local heating is on the tumor vasculature itself, or from secondary thermoregulatory responses in normal vessels (which are equipped by adequate neural-muscular regulation to recognize thermal signals) draining the heated tumor is not yet clear. Our data shows that mild, fever-range systemic hyperthermia also can significantly increase the percentage of perfused blood vessels within tumors and that this effect can last for hours. Moreover, systemic heating is associated with a significant depression in interstitial fluid pressure. Other recent research has also revealed that exposure of immune cells and tumor cells to mild hyperthermia have positive effects on the same immune mechanisms that are negatively impacted by hypoxia. Thus treatment with heat could have a two-pronged ability to improve anti-tumor immunity: reduced hypoxia-mediated immune suppression via heat-induced vascular changes in the tumor microenvironment and direct stimulation of anti-tumor immune mechanisms.

Many questions remain. If thermal regulation of blood flow is the key target for hyperthermia's ability to improve immunotherapy (or radiation or chemotherapy), are we using the optimal target temperature? The literature summarized here suggests that blood flow is highly sensitive to temperature, and that heat shock temperatures often aimed for in clinical protocols can actually damage blood vessels and/or inhibit blood flow. Moreover, would it be better to also heat normal vasculature surrounding a tumor in order to maximize thermoregulatory signals that increase blood flow to the tumor? Clearly, new detailed analyses on the differences between tumor and normal vasculature in terms of thermal sensitivity, thermoregulation and neural regulation are needed. Another question is raised when considering the types of tumor models which have been used by investigators who have studied hypoxia and the ability of hyperthermia to improve blood flow and oxygen tensions. Essentially all of the published literature utilizes transplantable tumors derived from long-term cell lines. Will these same effects be observed in spontaneous tumors in which both the tumor and the vasculature derived from the host? Moreover, how does hyperthermia affect oxygen tension and the blood vessel function in metastatic tumors? As was stated above, the clear benefit seen when hyperthermia is added to chemotherapy and/or radiation in actual patient tumors strongly suggests that there will be a positive effect on tumor vascular function and hypoxia status. But, many more studies are needed to truly assess the full potential of thermal therapy. A deeper understanding of the relationships among hypoxia, anti-tumor immunity and tumor blood flow regulation is necessary before we can achieve the most optimal thermal therapy strategies designed not only to improve radiation and chemotherapy, but also long-term immunological control of tumor growth or metastasis.

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Table 1

Immunosuppressive effects of the hypoxic tumor microenvironment.

References	Target Cell Type	Effect of hypoxia on the target cells	Outcome of hypoxia-induced modification
(62)	Tumor cells	Increased tumor cell shedding of MIC molecules through impaired nitric oxide signaling and decreased sensitivity to MK can CTL-mediated killing.	Tumor immune escape
(67)	Dendritic cells	Inhibition of CD40, MHC expression, and Th1-type cytokine production	Impaired DC maturation and cytokine production
(69)	Dendritic cells	Accumulation of extracellular adenosine leads to decreased antigen presentation and enhanced IL-10 secretion	Diminishes the capacity of DCs to initiate and amplify Th1 immune responses
(74)	T cells	Diminished IL-2 production	Impaired T cell growth and survival
(77)	T cells	Downregulation the expression and activity of Kv1.3 channels	Inhibition of TCR-mediated T cell proliferation
(78)	T cells	Inhibition of the accumulation of IL-2 and IFN-γ during TCR-driven differentiation of CTL	Less CTL development
(83)	T regulatory cells	Upregulation of cAMP-elevating A2A and HIF-1α to enhance the transcription of immunosuppressive molecules and extracellular adenosine accumulation	Beneficial for Treg cell development and increased anti-tumor immune suppression
(85)	T regulatory cells	Induction of Foxp3 expression through A2A-cAMP pathway and may enhance the expression of HIF-1α	Increased intensity of cAMP-elevating A2A and HIF-1pathway on Treg cells
(84)	T regulatory cells	Upregulation of Foxp3 expression in a HIF-1α dependent manner	Increased number and suppressive properties of naturally occurring CD4+CD25+ Treg cells.
(92)	Tumor-associated macrophages	Tumor cells secrete higher amounts of chemoattractants	Enhance monocyte attachment to and migration through the tumor vasculature and differentiate to TAM
(93)	Tumor-associated macrophages	Decreased mobility and inhibition of the chemoattractant signaling cascade	Entrap TAMs in the tumor hypoxic environment
(94)	Tumor-associated macrophages	Inhibition of the CCR5 chemokine receptor expression on macrophages	Immobilization of TAMs
(91)	Tumor-associated macrophages	Activation of a protumor phenotype in macrophages to promote tumor growth	Increased TAMs differentiation

Table 2

Heat-induced changes in tumor oxygenation.

References	Tumor Type	Site	Tumor oxygenation
(109)	C3H mammary tumor	Mouse leg (i.m.)	pO_2 increased at < 41 °C and decreased at > 42°C.
(110)	S-180 tumor	Mouse limb (s.c.)	pO_2 increased during heating at 41 °C for 30 min. pO_2 increased at 42°C for 30 min, decreased after heating, but increased again 14-18 hr after heating. pO_2decreased at 45°C for 30 min and failed to recover after heating.,
(111)	DS carcinoma	Rat foot	Oxygenation (Hb O_2 in blood vessels) peaked at 39.5°C and decreased at 42°C.
(74)	R3230 AC tumor	Rat leg (s.c.)	pO_2 increased during and 12-15 min after heating at 40.5-43.5°C for 30 min. pO_2 increased during and 12-15 min after heating at 40.5-41.5°C for 60 min but decreased during heating at 43°C for 60 min
(77)	R3230 AC tumor	Rat leg (s.c.)	pO_2 decreased during heating at 42.5°C for 60 min but increased 24 hr after heating pO_2 decreased during and 24 hr after heating at 43.5°C for 60 min
(85)	C3H mammary tumor	Mouse flank or leg (s.c.)	pO_2 increased 24 hr after heating at 43.5°C for 60 min. pO_2 decreased 4 hr after heating at 43.5°C for 120 min. Heat-induced changes in pO_2 depend on tumor site because pO_2 in the legs did not increase by heating.
(119)	SCK mammary carcinoma	Mouse leg (s.c.)	pO_2 increased during and 12-15 min after heating at 41.5°C for 60 min
(115)	Human soft tissue sarcoma	Human extremity	pO_2 increased I day after first hyperthermia