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COMMENTARY

THE HYPOXIC TUMOR CELL: A TARGET FOR SELECTIVE CANCER CHEMOTHERAPY

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The therapy of cancer is a multi-disciplinary problem, which involves the use of surgery, radiotherapy and chemotherapy as major treatment modalities. Each of these approaches has limitations. Surgery, which is a major curative modality for localized disease, cannot alone cure neoplasms which are widely disseminated or which have invaded critical normal structures. Radiotherapy, although useful in curing localized neoplasms and eradicating microscopic metastases, cannot be employed to control widely disseminated disease. Furthermore, there are certain clinical situations in which the success of radiotherapy is limited either by an extremely radioresistant neoplasm or by a tumor invading a very radiosensitive normal tissue. In either of these situations, it may be impossible to deliver a curative dose of radiation to the tumor without causing unacceptable damage to critical normal tissues. Significant advances toward the cure of human cancer by chemotherapy have been achieved, primarily with cytotoxic agents directed toward proliferating cells. Certain rapidly growing cancers, such as childhood tumors and Hodgkin's disease, respond dramatically to existing chemotherapy, and both localized and disseminated malignancies are frequently cured. However, these relatively responsive neoplasms represent only a small proportion of the malignancies that occur in man. Relatively slow-growing solid tumors, such as carcinomas of the lung, colon, and breast, constitute the majority of human cancers. These neoplasms, in general, respond poorly to existing chemotherapeutic agents, and curative treatment by any therapeutic modality or combination of modalities is uncommon in patients presenting with extensive disease. Slow-growing solid tumors constitute the major cause of mortality from cancer [1] and are responsible for many of the socioeconomic problems associated with human malignancies.

Considerable effort has been expended to identify biochemical characteristics unique to malignant cells which could be exploited in a therapeutic attack. Although a variety of metabolic differences between normal and neoplastic cells have been reported, few, if any, have proven to be either unique to cancer cells or useful in developing selective chemotherapy. The hypoxic cells in solid tumors are an obstacle to effective cancer treatment. Residual malignant cells, protected from radiotherapy or chemotherapy by hypoxia, may be capable of proliferating and causing the tumor to recur. This commentary examines the evidence for the occurrence of hypoxic cells in solid tumors and describes a conceptual approach for developing chemical agents which exploit the metabolic characteristics unique to cells in hypoxia, enabling selective destruction of these therapeutically resistant cells.

Importance of hypoxic cells in cancer therapy

Hypoxia has long been known to protect cells from the cytotoxic effects of radiation. Radiation dose–response curves for mammalian cells irradiated in air have slopes which are three times as steep as dose–response curves for cells irradiated under severely hypoxic conditions [2, 3] (Fig. 1). Solid neoplasms are known to contain deficient vascular beds, areas of severe vascular insufficiency and, often, regions of frank necrosis [4, 5], and therefore would be expected to contain hypoxic cells (Fig. 2).

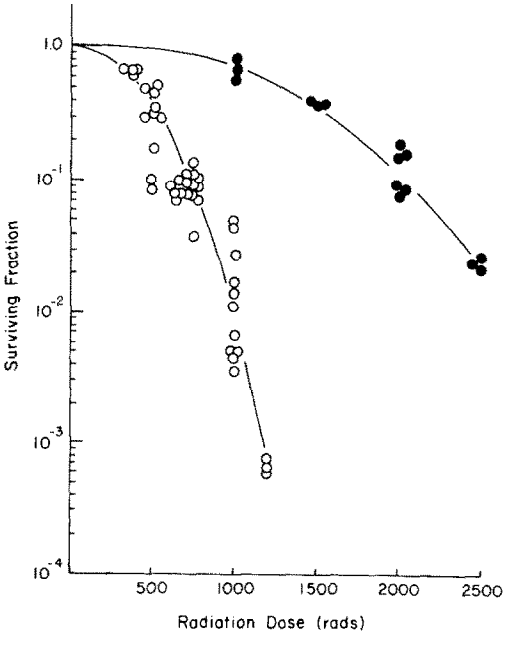

Fig. 1. Survival of EMT6 tumor cells irradiated *in vitro* under normal aeration (○) and severe hypoxia (●).

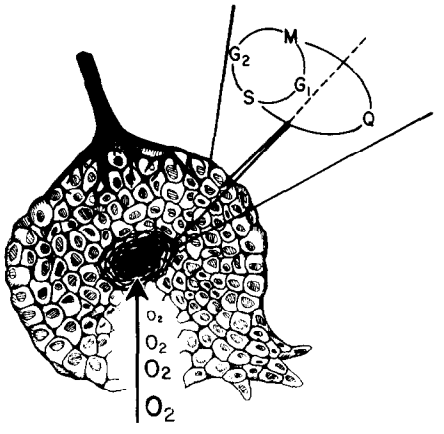

Fig. 2. Diagramatic representation of a solid tumor. This neoplasm is well vascularized on the surface by blood vessels extending from nearby normal tissues, and oxygen gradients exist within the tumor. Cells on the edge of the necrotic area, located in the center of the tumor, are remote from blood vessels, and are thought to be severely hypoxic, but viable. Both aerated and hypoxic cells are capable of progressing through the cell cycle, but more hypoxic cells than aerobic cells may be quiescent (Q) or may have prolonged cell cycle times.

In 1955, Thomlinson and Gray [5] analyzed the distribution of blood vessels, viable tumor tissue, and necrosis in pathologic specimens from human bronchogenic carcinoma in terms of the distribution and utilization of oxygen within the tissue. They concluded that the viable cells on the edge of necrotic regions in these tumors were probably severely hypoxic. The presence of hypoxic cells in solid tumors was first demonstrated radiobiologically by Powers and Tolmach [6] in 1963 using a transplantable mouse lymphosarcoma. Since that time, the oxygenation of several autochthonous and transplanted neoplasms in rodents has been studied. It now appears certain that the vast majority of these cancers contain hypoxic cells, which frequently constitute 10–20 per cent (and occasionally over half) of the total viable tumor cell population [7–9]. In fact, some solid rodent neoplasms contain as many hypoxic cells as cells in the S phase of the cell cycle [9–11].

Very small tumors, as well as large necrotic tumor masses, may contain hypoxic cells. Hypoxic cells have been shown to be present in transplantable mouse mammary carcinomas as small as 1 mm in diameter [10, 12, 13]. In culture, cells grown as spheroids (i.e. small balls of tightly packed cells) show central necrosis and radiobiologic evidence of hypoxia when they grow to sizes greater than 0.35 mm in diameter [14]. It is therefore quite probable that most occult metastases, too small to be detected by standard diagnostic techniques, contain foci of hypoxic cells, and that these hypoxic tumor cells are of major importance in determining the outcome of therapy for metastatic and microscopic disease (Fig. 3).

It has been shown repeatedly [3, 6–8, 11–13, 16–19] that the hypoxic cells in solid murine tumors limit the responses of these neoplasms to treatment with ionizing radiation delivered as a single fraction, and that the hypoxic cells surviving large doses of radiation are capable of either reestablishing the tumor *in situ* [11–13, 16–19] or producing tumors in other animals upon transplantation [3, 6, 11, 18, 19]. Fractionated radiotherapy of animal tumors results in a more complex situation because "reoxygenation" may occur between treatments [7, 8]. During reoxygenation, the tumor cells that were hypoxic at the time of irradiation reacquire the radiosensitivity characteristic of aerobic cells. As a consequence, the

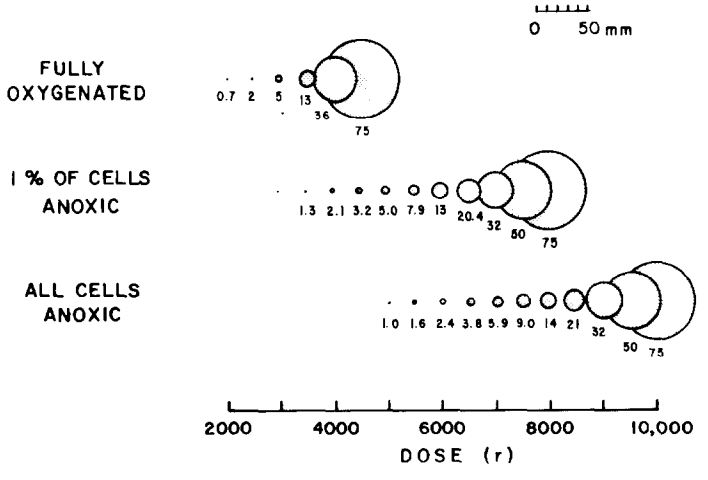

Fig. 3. Dose of X-rays necessary to give a 90 per cent chance of curing tumors of different diameters (as indicated below each mass). Top: all cells assumed to be well oxygenated. Bottom: all cells assumed to be hypoxic. Middle: 99 per cent of the cells assumed to be well oxygenated; 1 per cent, hypoxic. The presence of even a small proportion of hypoxic cells dramatically increases the radiation dose necessary to cure the tumors. (Redrawn from Ref. 15 with the permission of the author.)

sensitivity of the tumor to subsequent irradiation is increased. However, patterns of reoxygenation in animals vary with the dose and conditions of irradiation, and with the tumor line examined [7, 8, 20–24]. In some cases, tumors reoxygenate rapidly and extensively. However, in other instances, reoxygenation is relatively slow, and hypoxic cells continue to be a problem during fractionated radiotherapy.

Two other observations illustrate the importance of hypoxic cells as a source for tumor regrowth after treatment. First, it has been found that transplantation of tissue from necrotic regions of tumors can produce malignancies with a relatively high frequency [25]. Second, fragments of Walker 256 carcinosarcoma which had been perfused for 56 hr with medium low in oxygen were shown to grow as well as transplants from well-oxygenated tumor tissue [26]. These observations are consistent with experiments demonstrating that cells in culture remain viable, and may even continue to proliferate for considerable periods of time under conditions of moderate or severe hypoxia [27, 28].

Techniques for the direct measurement of the proportion of hypoxic cells in human tumors are not yet available; only indirect evidence has been obtained for the presence of hypoxic cells in these neoplasms. Measurements of oxygen concentrations in human tumors and normal tissues using an O_2 electrode have revealed that the oxygen concentrations in solid human tumors are lower than those in normal tissues [29]. However, because of technical limitations of the methodology, these experiments measured average oxygen concentration on a macroscopic scale and could not establish whether human tumors possess regions of severe hypoxia containing viable cells. Dose–response curves for therapy of superficial skin carcinoma with large single doses of radiation suggest the presence of hypoxic cells in human tumors [30], as do the results of irradiation of certain human malignancies with short courses of high dose radiotherapy combined with either hypoxic cell sensitizers [31–33] or hyperbaric oxygen [34, 35].

The importance of hypoxic cells in limiting the sensitivity of human tumors to conventional, fractionated radiotherapy regimens is still debated extensively. Some clinical data [30, 35] and extrapolations from some animal tumors in which reoxygenation occurs quite slowly [8, 21, 22] suggest that hypoxic cells probably limit the curability of certain types of human cancer by conventional, fractionated radiotherapy.

The selectivity of many chemotherapeutic agents in current clinical use for the treatment of cancer appears to derive from the toxicity of the drugs to those cells which are actively transversing the cell cycle. The toxicity of such agents to proliferating cells of normal tissues limits the clinical usefulness of these drugs. Cycle-active agents are relatively ineffective against quiescent tumor cells which are not actively cycling at the time of treatment but are capable of commencing proliferation at a later time and causing the tumor to regrow. Quiescent cells (Fig. 2) exist in both non-human animal and human tumors [8, 9], and the quiescent cells of non-human animal tumors are "clonogenic" (i.e. capable of indefinite proliferation) [8, 36]. Hypoxic cells are resistant to conventional chemotherapy. These cells may have prolonged cell cycle times [28] or may be blocked in their progression through the G_1 phase [27]. Thus, hypoxic cells would be expected to be relatively resistant to any chemotherapeutic agent directed toward proliferating cells. Hypoxic tumor cells also may be resistant to chemotherapeutic agents due to pharmacodynamic considerations. For example, appropriate concentrations of drugs that have physico-chemical properties not conducive to diffusion into tumor tissue, or that are unstable or metabolized rapidly may not reach chronically hypoxic tumor cells located in regions of severe vascular insufficiency.

Bioreductive activation as a basis of selective cytotoxicity

In 1972, it was hypothesized that hypoxic cells remote from the vascular supply of a tumor mass might have a greater capacity for reductive reactions than their normal well-oxygenated counterparts [37]. It had been demonstrated in the 1930's that anaerobic cultures of microbes had a lower half-wave potential (i.e. a greater capacity for reduction) than did aerobic cultures. As cultures under either aerobic or anaerobic conditions grew and became more crowded, the redox potential of the microbial cultures decreased [38, 39]. By analogy, hypoxic cells in solid tumors may exist in an environment conducive to reductive processes. It was thought that this characteristic of hypoxic cells might be exploited by developing chemotherapeutic agents which became cytotoxic after reductive activation [37].

Two classes of agents are presently known which exhibit preferential cytotoxicity toward hypoxic cells through reductive activation: (a) the quinone bioreductive alkylating agents, a naturally occurring prototype of which is mitomycin C, and (b) the nitroaromatic heterocyclic hypoxic cell sensitizers, such as misonidazole and metronidazole. The first bioreductive alkylating agents designed and synthesized by Lin *et al.* [37, 40–45] were a series of benzo- and naphthoquinones. These compounds were hypothesized to be activated preferentially by hypoxic cells to produce highly reactive quinone methides which would alkylate cellular elements. The nitroheterocyclic radiosensitizers were first examined by radiobiologists because of their ability to sensitize hypoxic cells preferentially to the cytotoxic effects of ionizing radiation [33]. It was later discovered [46–53] that these compounds were also selectively toxic to hypoxic cells and that the toxic metabolites were the result of reductive activation by these cells [54].

Mitomycin C and other quinones

Bioreductive activation may well be important to the expression of cytotoxic activity by several existing antineoplastic agents. It is conceivable that quinones, such as mitomycin C, adriamycin, daunorubicin and streptonigrin, produce reactive species upon reduction of the quinone moiety [55–61]. We have investigated this possibility with the mitomycins, a group of structurally related antibiotics which are toxic to both bacterial and mammalian cells.

Early studies demonstrated that mitomycin C and

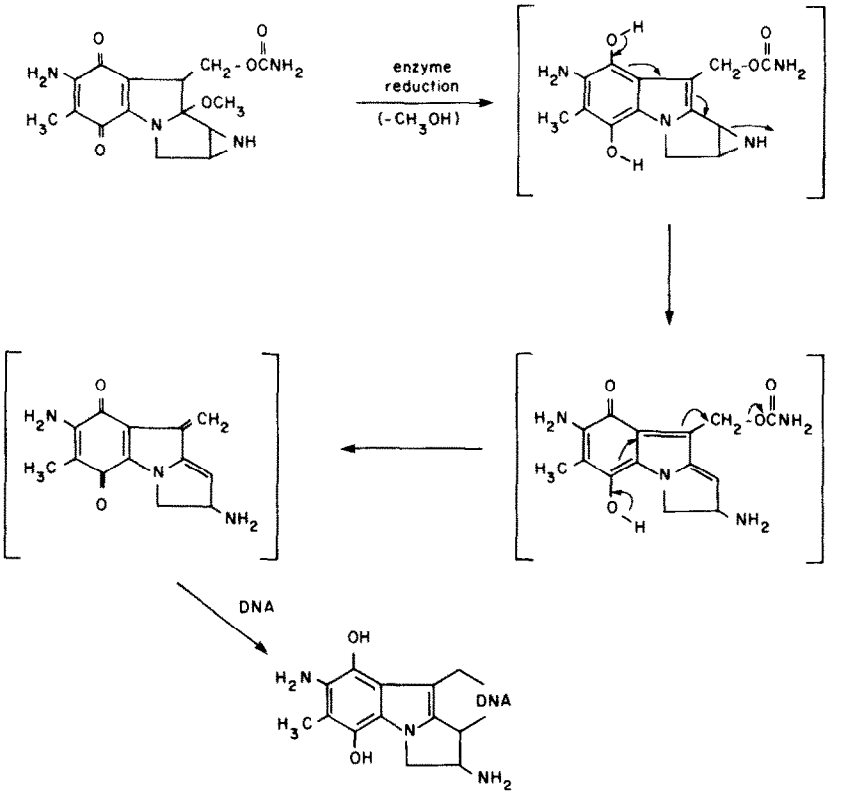

Fig. 4. Activation of mitomycin C to an alkylating agent. After enzymatic reduction of the quinone group, the elimination of the methoxy and carbamyl groups and the opening of the aziridine ring occur spontaneously to generate the hypothesized quinone-methide intermediate, which is extremely reactive.

its analogs act as bifunctional alkylating agents, which can form DNA–DNA and DNA–protein crosslinks. Reduction of the benzoquinone ring of the mitomycin molecule (as shown in Fig. 4) is a prerequisite for alkylation of DNA [59]. Studies of the structural requirements of mitomycin molecules have suggested that the carbamyl and aziridine moieties were not strictly essential for activation [62, 63] and that antineoplastic activity was correlated with low redox potential [64]. The results of these studies suggested that simple benzo- and naphthoquinones containing one or two sites open to nucleophilic attack after reduction of the quinone moiety might function as bioreductive alkylating agents [55, 56, 65, 66]. Several such compounds were synthesized and shown to have significant activity against Sarcoma 180 [40–44, 65, 67, 68]. Studies on the relationship between the oxidation–reduction potential of these benzo- and naphthoquinone derivatives and their effectiveness as antitumor agents revealed a positive correlation between redox potential and antineoplastic activity [41, 68, 69]; agents having the lowest redox potentials were the most efficacious. It is probable that an optimal oxidation–reduction potential exists, at which the antitumor activity of bioreductive alkylating agents is maximal. Because mitomycin C, the most efficacious of the quinone bioreductive alkylating agents tested by this laboratory to date, also has the lowest redox potential, the design and the preparation of new compounds with even lower redox potentials are warranted.

Early studies on the biotransformation of mitomycin C demonstrated that an NADPH-dependent enzyme system located in liver microsomes was involved in the metabolism of the drug [57] and that in bacterial lysates an NADPH-dependent enzyme(s) was essential for the activation of mitomycin C to an alkylating species [59]. Studies in our laboratory have confirmed and extended the original observations of Schwartz [57] and Iyer and Szybalski [59]. We found that mitomycin C was bioactivated under hypoxic conditions in liver microsomes and nuclei by an NADPH-dependent enzyme(s) [70]. The enzyme system was inhibited both by oxygen and by carbon monoxide. Furthermore, using 4-(*p*-nitrobenzyl)pyridine as a trapping agent for activated drug, we found that manipulations which inhibited the metabolism of mitomycin C also interfered with the generation of alkylating species. The metabolism of mitomycin C in two tumor cell lines, Sarcoma 180 and EMT6 carcinoma, was examined using techniques similar to those employed for studying the liver-dependent metabolism of the drug [71]. Both of these neoplastic cell lines were able metabolically to activate mitomycin C to an alkylating agent under hypoxic conditions; the enzyme system appeared to be similar to that in the liver.

Although mitomycin C is selectively activated to an alkylating species under hypoxic conditions, the clinical use of this agent has been limited by its severe toxicity to normal tissues. Presumably, toxicity to well-oxygenated tissues is the result of dif-

ferent activation mechanisms, one of which involves single electron reduction followed by reoxidation of mitomycin C by molecular oxygen to give the parent compound and the superoxide radical [72–74]. The superoxide free radical can dismute to hydrogen peroxide and to other radicals, such as the hydroxyl radical, which are cytotoxic. The toxic manifestations of superoxide radicals, hydroxyl radicals, and hydrogen peroxide have been reviewed elsewhere [75, 76]. Preliminary experiments in our laboratory have demonstrated that mitomycin C at relatively low concentrations is more cytotoxic to chronically hypoxic cells than to cells maintained under oxygenated conditions [71, 77]. These findings suggest that the clinical employment of mitomycin C at maximum tolerated doses will result in a loss of specificity of this antibiotic for hypoxic cells, and that mitomycin C could be given to cancer patients at low doses to selectively damage hypoxic cells with minimal induction of oxygen-dependent cytotoxicity. Such treatments might be extremely valuable in combination with radiotherapy or chemotherapy directed against well-oxygenated tumor cells. It is also possible that compounds could be developed which can be enzymatically bioactivated by hypoxic cells but which are not amenable to the aerobic reduction that leads to the superoxide-mediated toxicity observed with mitomycin C in well-oxygenated cells. Such agents would be toxic exclusively to hypoxic cells. Experiments to study this possibility are in progress.

Nitroheterocyclic compounds as hypoxic cell specific agents

The nitroheterocyclic radiosensitizers, such as metronidazole and misonidazole, increase the sensitivity of hypoxic cells to ionizing radiation. Radiation produces a variety of lesions in DNA, including scissions, crosslinks, adducts and chemical alterations of the bases and sugars. Damage to DNA results directly, from the interaction of the radiation with DNA, or indirectly, from chemical reactions between DNA and superoxide or other radicals produced by the interaction of the radiation with water and other cellular components [78]. Oxygen alters the number and character of the free radical reactions, and therefore increases the DNA damage produced by a given dose of radiation. As a result, the slope of the cell survival curve for aerobic cells is increased by a factor of approximately 3 compared with that for severely hypoxic cells (Fig. 1). Radiosensitization occurs at relatively low concentrations of oxygen: a concentration of 0.25% oxygen moves the dose–response curve half-way toward the fully aerated condition, while essentially identical dose–response curves are obtained for cells in 2, 20, or 100% oxygen. The nitroimidazole compounds are thought to sensitize hypoxic cells to radiation by interacting with electron excess or free radical centers [79] to form nitroxyl free radical anions (Fig. 5). As a consequence, these compounds sensitize hypoxic cells to radiation; they do not sensitize aero-

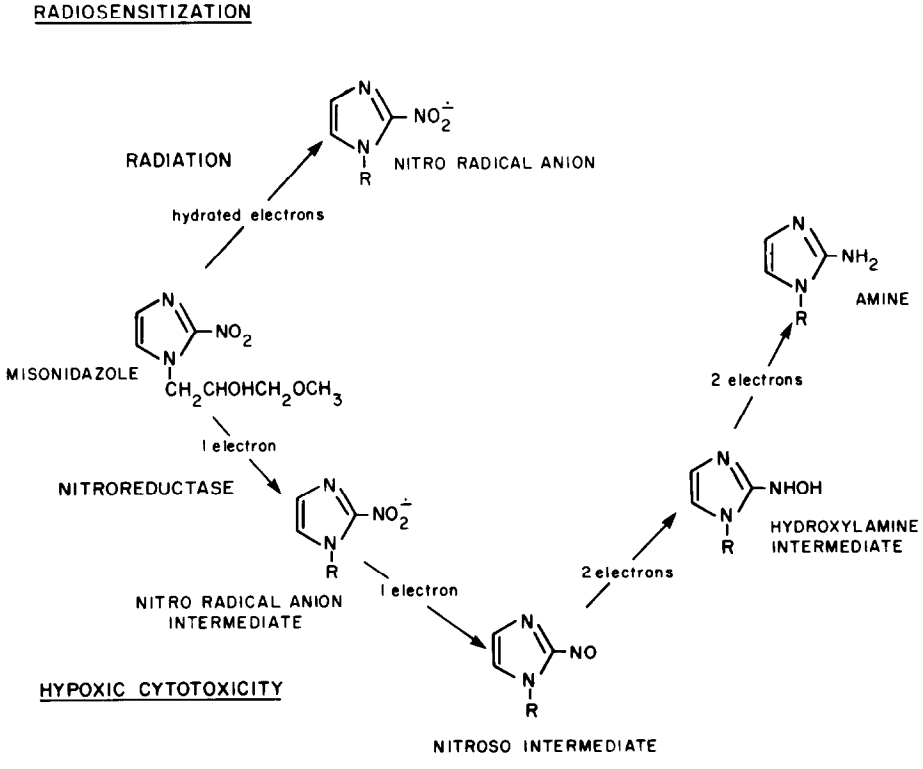

Fig. 5. Activation of the radiosensitizer, misonidazole. The first step in either radiation-induced activation or metabolism by nitroreductases leads to the production of a nitro radical anion.

bic cells, which are already fully radiosensitive.

The nitroimidazole radiosensitizers have been shown recently to be selectively toxic to hypoxic cells *in vitro*, whereas well-oxygenated cells were protected from misonidazole-induced cytotoxicity [47, 48]. The severity of the hypoxia determined the susceptibility of cells to misonidazole-induced cytotoxicity, as assayed by colony formation [47]. In addition, the metabolism of misonidazole was different in oxic and hypoxic cells. Relatively large amounts of *N*-hydroxy and amine metabolites were produced in hypoxic cells (Fig. 5), indicating that reduction of the nitro group occurred [47, 48, 80]. Few metabolites were found in oxic cells [48]. The selective cytotoxicity of misonidazole to hypoxic cells appeared to be the result of the generation and retention of misonidazole metabolites [48, 80–82]. These findings indicate that cells maintained *in vitro* metabolize misonidazole to reactive forms, and that the metabolic pathways are dependent on the state of cellular oxygenation.

Studies employing chemical reduction of misonidazole or metronidazole have substantiated the concept that reduction of the nitro group can lead to reactive species capable of interacting with macromolecules [83, 84]. Chemical reduction of metronidazole in the absence of oxygen produced intermediates that reacted with calf-thymus DNA [83, 84]. Sulfhydryl reagents, such as cysteamine, protected against the cytotoxic effects of these compounds, supporting the hypothesis that reactive intermediates (i.e. the nitro radical anion or the nitroso intermediate) were the cytotoxic species [85]. In support of these findings, incubation of $CH2B_2$ cells with metronidazole or misonidazole under hypoxic conditions resulted in substantial time-dependent damage of DNA, measured as alkali labile lesions, which were interpreted as single strand breaks [86]. Electrolytic reduction of metronidazole under anaerobic conditions in the presence of calf-thymus DNA caused a loss of the helical content of DNA as well as strand breakage [87].

Although the enzymatic systems responsible for the reduction of nitroimidazoles in mammalian tumor cells have not been well characterized, much has been learned about nitro group reduction in liver microsomes. Nitro-reductase activity in hepatic microsomes is dependent upon either NADH or NADPH, inhibited by oxygen, and stimulated by flavins [88–90]. Complete reduction of the nitro group requires a six-electron transfer from pyridine nucleotides and results in the formation of a primary amine [89]. Because microsomal flavins and cytochromes are one-electron donors, the first step in the reduction would be the formation of the nitro radical anion [91] (Fig. 5). Many aromatic and aliphatic nitro compounds interact with dithionite reduced hepatic microsomal cytochrome P-450-Fe(II), and an unstable intermediate (most likely the nitroso compound which is isoelectronic with dioxygen) is produced *in situ* during reduction of the nitro compound [92, 93]. The formation of these nitroso complexes is not limited to cytochrome P-450; complexes can occur with other hemoproteins presenting access to the heme pocket, such as cytochrome P-420, hemoglobin and myoglobin [93]. Further reduction of the nitroso and hydroxylamine compounds could also occur. Studies on the reduction of *N*-hydroxyphentermine have shown that this material is reduced by at least two enzyme systems, one of which is inducible by phenobarbital, requires NADPH, and is sensitive to carbon monoxide [94]. It is probable that similar enzyme systems exist in tumor cells that allow the cells to reductively activate the nitroimidazoles to reactive, cytotoxic compounds.

Other approaches for attacking hypoxic cells

Hypoxic tumor cells and the well-oxygenated cells of both tumors and normal tissues probably differ in many other features of their physiology and metabolism, and it may be possible to employ these differences in selective therapeutic attacks. For example, cells *in vitro* are more susceptible to the cytotoxic effects of hyperthermia when rendered hypoxic by crowding and metabolic depletion than when growing normally in suspension or monolayers [95]. The increased sensitivity of hypoxic cells to hyperthermia is probably related to alterations in environmental pH [96] and nutritional status [97] and is thought to be mediated through changes in the structure and interactions of the cell membrane [98].

The glucose analogue, 5-thio-D-glucose, is more toxic to P185-X2 mastocytoma cells *in vitro* during hypoxic incubation than during aerobic incubation [99]. Differences in the energy metabolism of the cells under hypoxic and aerobic conditions may be responsible for this increased sensitivity. The growth of P815-X2 mastocytoma *in vivo* [99] is also inhibited by 5-thio-D-glucose.

Such findings suggest that physiologic, metabolic, and environmental alterations accompany hypoxia in solid neoplasms. A systematic examination of the characteristics of hypoxic cells in solid cancers should be undertaken to determine whether other metabolic characteristics specific to hypoxic cells are amenable to manipulation in a therapeutic attack on solid tumors.

Conclusions

Hypoxic cells may limit the response of tumors to conventional radiotherapy because of their inherent radioresistance. Furthermore, the hypoxic cells of solid neoplasms may be unresponsive to conventional chemotherapy. The proliferation patterns of hypoxic tumor cells probably differ from those of their well-oxygenated counterparts, in that many hypoxic cells are non-cycling or are cycling with prolonged and abnormal cell cycle times. Such cells would have reduced sensitivities to cycle-active chemotherapeutic agents. Moreover, hypoxic cells are found in regions of vascular insufficiency and, therefore, may not be exposed to appropriate concentrations of agents which have limited diffusion through tissue or which are rapidly degraded to inactive species. Agents active against hypoxic cells would be expected to attack malignant cell populations which are not adequately destroyed by existing therapeutic modalities. Because hypoxic cell populations occur even in very small solid tumors, while normal body tissues are generally well oxy-

genated, such agents would be valuable in treating both primary and metastatic disease.

Hypoxic cells in solid tumors exist in an environment conducive to reductive processes and are differentially sensitive to the cytotoxic actions of drugs which require prior reductive activation. The presently available bioreductive alkylating agents represent a first step toward the development of drugs directed against hypoxic cells. One agent of this class, mitomycin C, may have increased clinical utility if employed in regimens in which low dose therapy with mitomycin C to achieve selective kill of hypoxic cells is combined with concurrent radiotherapy or chemotherapy designed to eradicate well-oxygenated cells. It is possible that bioreductive alkylating agents could be developed which would lack the aerobic cytotoxicities of existing compounds, which probably result from metabolic processes different from those leading to cytotoxicity in hypoxic cells. Therefore, these drugs should exhibit minimal toxicities to normal body tissues, which are, for the most part, well oxygenated. It is also possible that new nitroaromatic heterocyclic agents could be developed which maintain the selective metabolic reduction and differential cytotoxicity of the nitroimidazoles, but which are more cytotoxic and, therefore, more useful as chemotherapeutic agents. Results obtained with bioreductive alkylating agents, glucose analogues, hyperthermia, and hypoxic cell radiosensitizers indicate the need for further research to elucidate the biological properties of hypoxic cells in solid neoplasms; this may ultimately lead to the development of new agents capable of a selective chemotherapeutic attack on solid tumors.

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