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Amplified Hypoxia Induced Tumor-Cell Death *In vitro*

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Abstract

Cancer is the state of cells marked by unregulated proliferation. There are several characteristic features attributed to its occurrence so as to demarcate itself from the normal tissue system, and one of them is Angiogenesis, that ensures its continued survival in the body. Angiogenesis, in the recent past has become a nucleus of notice and has hence opened new portals in the realm of cancer therapy. Anti-Angiogenic studies have made hysteric escalations in the evolution of effectual anti-cancer drugs. This review, in an attempt to contribute to the contemporary therapies, talks about a novel therapeutic approach of targeting tumor cells by depriving oxygen from them through increased hypoxia which surpasses their minimum requirement for oxygen; the latter achieved by interfering at the interface of oxygen diffusion between the blood vessels and tumor cells.

Introduction

Angiogenesis is a physiological process which entails the growth of new blood vessels from pre-existing vessels. It internally mediates nutrition of the tumor cells by supplying oxygenated blood drawn from the systemic circulation and disseminated to the tumor tissues via special blood vessels called-Micro vessels; the specific name is due to its extremely thin diameter. This simple membrane transport procedure involves the diffusion of oxygen from the endothelial wall of micro-vessels into the tissue fluid around tumor cells and thence, to the cells of the tissue.

Now the *Single Hit Therapy*, which this review elaborates on, is primarily based on certain important conceptions that are discussed in the following passages. A cancerous cell thrives on only 65% oxygen in comparison to a normal cell or in other words taking away, from a cell, 35% of its oxygen for 48 hours will render it cancerous by Warburg. An alternate correlation to this finding is the thin diameter of the tumor tissue microvessls. At the molecular level this deficient state of oxygen activates Hypoxia Inducible Factor, a transcription factor that stimulates the release of Vascular Endothelial Growth Factor (VEGF), an important signaling protein involved in angiogenesis. A search for DNA control sequences, which influence gene expression regulated by net oxygen content in their ambiance, led to the identification of a powerful regulatory element lying 3´ to both the human and murine genes (the Epo 3´ enhancer) by Beck, Pugh, Semenza. Detailed studies of this enhancer defined several binding sites, one of which was critical for hypoxia-inducible function and bound a complex termed Hypoxia- Inducible Factor-1 (HIF-1) by Semenza and Wang. HIF-1, when stabilized by hypoxic conditions, up regulates several genes to promote survival in low oxygen conditions.

In addition to this, it has been interestingly noticed in some of the early works by Papandreou that although HIF transcription is activated during hypoxic conditions in tumor cells, an oxygen concentration as low as 0.01% triggered apoptotic events in tumor cells, characterized by decreased colony formation, chromatin condensation, DNA fragmentation, and caspase activation; these changes, however, were independent of HIF status (both HIF proficient and deficient tumor cells) in tumor cells.

Hence, the above observations provoke an intriguing postulation according to which, even though HIF helps a tumor cell cope up with hypoxic condition, there probably exist a minimum threshold level of net oxygen content which the Hypoxia Inducible Factor requires to be able to sense and stimulate pathways for angiogenesis. There is perhaps an upper limit for HIF activation below which the canonical pathway leading to its (HIF) expression is not triggered thereby being unable to help a tumor cell out of its hypoxic state through the process of angiogenesis that it activates. During the hypoxic phase, tumor cells depend on pathways like glycolysis to meet their energy requirements until they are ready with blood vessels to extract energy from aerobic cell respiration. The energy production in tumor cells, under insufficient oxygen, and blood supply, is looked after by HIF-1.HIF-1 influences the recruitment of enzymes vital for glycolysis, in the tumor cells; so as to meet the insufficient supply of oxygen necessary for oxidative phosphorylation in the mitochondria by Iyer and Seagroves. But what must not be forgotten is that it is not a permanent alteration. It is basically to ensure that the tumor tissue meets its energy requirements until angiogenesis; because HIF, at some stage also stimulates the transcription of genes encoding the Vascular Endothelial Growth Factor (VEGF) by G.L. Semenza and T. Hofer. Secondly, the use of glucose, via glycolysis, to generate ATP is only applicable if the glucose is in excess; hypoxic tumor cells under low glucose have been found to undergo cell death by Papandreou. Increase in glycolysis, in tumor cells under low oxygen tension, has been associated with decrease in mitochondrial respiration by Papandreou. The decrease in respiration is due to the reduced transport of pyruvate, by the activity of pyruvate dehydrogenase kinase, into the TCA cycle.

So by making tumor cells more hypoxic, by reducing the concentration of oxygen below what HIF-1 can sense by Papandreou, we end up rendering HIF-1 senseless. As a result, of which the cells will stay in a prolonged state of hypoxia. The energy production via glycolysis cannot solely meet all the ATP needs of the tissue, then. Meanwhile, release of cytokines, usual in a tumor cell under hypoxic state, will lead to the production of Nitric Oxide (NO) by T. Hagen. This will further inhibit HIF activity by release of reactive oxygen

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species from mitochondria; this reinstates prolyl hydroxylase activity which mediates HIF degradation via ubiquitination by C. Riganti. Such an energy deficient situation, combined with NO mediated inhibition of electron transport chain complexes by T. Hagen, causes a shift in the energy metabolism especially in the mitochondria, there by activating factors that force it towards an immediate fragmentation which marks the onset of apoptosis, as fragmentation induces release of intrinsic pro-apoptotic factors like cytochrome-c by D. Arnoult. Thus if one sums up the abovementioned concepts it becomes evident that, even though an oncogenic cell requires less oxygen than a normal cell, it certainly does call for a certain minimum amount. For this reason any further dispossession of this minimum oxygen will have drastic effects on it.

It is a known fact that before the diffusion of oxygen into the deoxygenated tissues the former is brought by hemoglobin as a complex of Ferrous (Fe)-Oxygen (1:1). The *Single Hit Therapy*, which is based upon the fundamentals of coordinate chemistry, proposes that the presence of a coordinate metal ion with an affinity for oxygen much greater than ferrous may sequester oxygen, during its dissociation from ferrous at the endothelial-tissue interface, and may consequently prevent the diffusion of free oxygen molecule through the endothelial wall into the tissues (tumor). The basic in coordinate chemistry which supports the aforesaid is:-

Smaller, the ionic radius of a cation or a central metal ion in a coordinate complex, more stable and strong is the bond it forms with a ligand. This conception can be further illustrated from the following table which shows the order of decreasing ionic radii among transition (d-block) elements.

ELEMENT	Fe^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}
IONIC-RADII (pm)	76	74	72	72

Hence, it's quite clear that since copper has a smaller ionic radius than iron, it may therefore form a better complex with oxygen in contrast.

Efficacy of a coordination complex of copper with oxygen

Usually, where most organic compounds are unreactive to molecular oxygen owing to its triplet ground state, transition metals and their ions which have multiple spins and oxidation states have no hindrance in reacting with oxygen. But such complexes also lead to the formation intermediate isolable oxygen adducts. Following which the reactivity of the coordinated oxygen are enhanced, especially towards organic compounds. Metal superoxide complexes are one of the major oxygen adducts. Situation of this nature will certainly post a huge problem in the formulation of a drug with the suitable coordinate central metal ion (in this case, copper). But there is an alternate observation, according to which, the addition of certain additional ligands to the metal center (e.g. addition of 2,2', bipyridyl to cobalt centers) can coordinatively saturate the metal ion; by doing so, the catalytic reactions leading to formation of adducts could be prevented by R.P. Hanzlik and Dale Williamson. Secondly, the interaction of transition metals with oxygen is sensitive to the oxidation potential of the metal complex by M.J. Carter. Thus increasing the electro negativity of the ligand could increase the oxidation potential of the complex, thereby reduce the propensity of complexes to undergo further catalytic reactions by R.P. Hanzlik and D.F. Smith. one of the suitable examples of the aforesaid come from the fact that addition of lithium ions (Li^+) to some of the cobalt complexes enhances the complex's oxidation potential and renders it without any catalytic activity. The inductive effects (The ability to push or attract electrons away from or towards itself) of bound Li^+ make the electron density on cobalt less available by M. Truter and C. Floriana. Among the other transition (d block) elements, Nickel and Cobalt seem less likely because they have been officially classified as carcinogens and possible carcinogens to humans (Group 1 and Group 2B) by the International Agency for Research on Cancer (IARC) in 1990. This notion, mentioned above, is merely theoretical in its architecture. However a distant possibility also refuses to go away.

Standards of oxygen diffusion and transport

It is imperative to understand the dynamics of tissue oxygen and supply, when conjuring up a therapeutic against tumors, especially one involving induction of hypoxia; because the very arrangement of tumor microcirculation has a major impact on the efficacy of anti-cancer measures. A simple process of diffusion mediates the transport of oxygen from the microvessels to the tumor tissues. The diffusive flux, here, is proportional to the gradient of the tissue partial pressure of oxygen (PO_2). As a result the local value of PO_2 at any given point, in the tissue, is reliant on the surrounding microvessel. PO_2, varying at different points, is determined by the interaction between the local supply of oxygen, owing to the microvasculature and the consumption by T.W. Secomb. Thorough experimentation confirms that the microcirculation of tumors differs from that of the normal tissues by Dewhirst. Reports suggest that by T.W. Secomb oxygen consumption as low as 0.1 $cm^3 0_2$/100g/min is enough to well oxygenate a central tumor region. However, increasing the consumption to 0.23 $cm^3 0_2$/100g/min leaves regions with localized hypoxia. The peripheral tumor region, in comparison to central tumor region can sustain higher oxygen consumption, due to higher vascular density in the former. In addition to vascular density the concept of diffusion distance is also useful in determining the extent of oxygenation in tissues with irregular vascular geometry (e.g. tumors) this is because the distance from tissue to the nearest vessel varies, largely; thereby rendering some regions with oversupply and others with undersupply by T.W. Secomb. Interstingly, there are differences in oxygen release between microvessels of same diameter by N. Tateishi. This has been attributed to the dissimilarity in oxygen diffusion and properties of the vessel wall. A pH gradient or a temperature gradient formed between the inside of the microvessel and the mesentry leads to a variation in the rate of oxygen release; a decrease of 27% was observed with a temperature changed from 30°C to 15°C by N. Tateishi. It has been also recognized that the dissociation of oxygen from hemoglobin occurs in several tens of a milliseconds. Also the volume fraction of tumor microvessels is minute in comparison to those oxygenating normal tissues by T.W. Secomb. This indicates that the time required by oxygen to diffuse into tissues is very less. Devising a drug, as mentioned earlier, will have to be done keeping this piece of information in mind.

Literature review

Many analogous works have been done in the recent past, some of which have been cited below: (Enhancement of Hypoxia-Induced Tumor Cell Death In vitro and Radiation Therapy In vivo by Use of Small Interfering RNA Targeted to Hypoxia-Inducible Factor-1-alpha, Xiuwu Zhang, Takashi Kon, He Wan); but they were intended towards preventing Subcutaneous tumor growth by down-regulating HIF-1 (alpha), for therapeutic gain, by use of small interfering RNA technology in combination with ionizing radiation. Although the results provided proof for an effective anti cancer drug, one must realize that the main drawback using radiation and chemotherapies

are that they specifically act on all fast growing cells, whether it is a tumor cell or a human hair.

Other works such as (Hypoxia-mediated Apoptosis from Angiogenesis Inhibition Underlies Tumor Control by Recombinant Interleukin12, Michael S. Gee, Cameron J. Koch, Sydney M. Evans) were carried out where, the role of angiogenesis inhibition in the antitumor activity of recombinant murine interleukin 12 (rmIL-12) was studied in K1735 murine melanomas, the growth of which was rapidly suppressed by rmIL-12 treatment. This resulted in tumor ischemia from therapeutic angiogenesis inhibition. *In vitro* studies indicated that the degree of hypoxia present within treated tumors was sufficient to trigger K1735 apoptosis, prevalent in the first week of rmIL-12 treatment.

Hence the current idea has not yet been exclusively undertaken in any of the works mentioned above and some others that have not been brought up in this review, thereby opening up opportunities for this new thought.

The Novel approach

This new outlook will aim at augmenting hypoxia in tumor cells by choking its aerobic tissue respiration, but this appears easier said than done; answers to certain imperative questions, which are key to the above stated , such as specific line of attack towards tumor cells and the supporting modes of approach, lie with a group of special cell surface receptors found on all endothelial cells called the VEGF*R* (Vascular-Endothelial Growth Factor *Receptors)*, to which aforesaid VEGF binds and trigger various biosignalling pathways towards angiogenesis.

A synthetic (hypothetical) drug containing the desired coordinate metal (*copper*) ion to bind with oxygen and which is distinctively designed to bind to the VEGF receptors, may serve the requisite purpose. The designing depends on the use of various drug designing computational tools to devise the specific Target (Lead) Molecule complementary to the receptor. The core intend here is to allow the drug with metal ion to be activated only when it binds to the cancer cell specific receptors so as to release the encircled metal ion into the region of the endothelial wall of the micro vessels in the inter-tumor region and serve the required purpose. The aforesaid skeleton for the novel approach has been elaborated as follows; each giving an in depth information on the Methodology behind designing a specific ligand (peptide) analogue that binds Vascular Endothelial Growth Factor (VEGF) receptors. Before, the designing of a specific peptide (VEGF) analogue, there are several considerations to be kept in mind. Firstly, these peptide analogues should be competitors to natural ligands at the receptor. Secondly, a drug design based on the 3D structure of the target receptor is usually unattainable because it is difficult to obtain the structures of some of the receptors. So nowadays, almost in all cases, homology modeling and site specific mutagenesis are being used very widely to design ligand agonists or antagonists by V.J. Hruby. The common strategy to design ligands, involve knowing the structural and conformational feature of peptides, in question (in this case VEGF); a detailed analysis of its (ligand) bioactivities and whole animal assay by V.J. Hruby, J. Rizo and Marshall G.R. The peptide ligand to be used as an antagonist should interact with the active site without a response; which can, then, be evaluated by secondary messenger assays by V.J. Hruby. The structure–activity relationship (SAR) or pharmacophore is an important characteristic feature of ligand design, either an agonist or an antagonist. Monoclonal antibodies (MAbs) against VEGF receptors are gaining equal importance, as effectual anticancer agents. A number biotechnological products based on MAbs, namely Avastin by Genentech/Roche, and Eribitux by Bristol-Meyers Squibb/ Merck KGA have found place in the market, for their intended use Biojob Blog 2008.

Design of VEGF Analogues: Among all the members of the VEGF family, VEGF-A, B and Placenta like Growth Factor (PlGF) are mainly required for the formation of blood vessels or angiogenesis by Alberts. The basic premise behind designing VEGF analogues is to ensure that the modified ligands exhibit more binding affinity for one or more VEGF receptors by Szkudlinski. The Kinase domain receptor or KDR for angiogenic signaling by Barleon. Some of the most important considerations kept in mind while synthesizing VEGF analogues are as follows:- The analogues must demonstrate at least about three to four enhancement in receptor binding affinity. The VEGF antagonists must be modified homodimers or heterodimers, wherein the molecules contain at least one mutation which might be present in one or both subunits of the VEGF molecule. These substitutions at several amino acid positions, namely 121,145,148,165, 183,189 or 206, could conjure up different VEGF-A isoforms by Szkudlinski. There are alternate amino acid substitution positions; these include, mostly, the basic amino acids like lysine and arginine by Szkudlinski. It is also important that VEGF analogues exhibit a decrease in bioactivity as opposed to the wild type ligand. Recent work by Szkudlinski show that an analog of $VEGF_{165}$ shows decreasing bioactivity in comparison to the natural ligand; as measured in the in vitro cell viability assays of endothelial cells. So in essence, the receptor binding affinity should be more and the bioactivity should be less, in analogues of VEGF. Later, large peptide libraries are made using combinatorial methods by N.K. Terret and V.J. Hruby. This is followed by High Throughput Screening (HTS), where these large libraries are screened to get the lead molecule. The screening checks how selective are the compounds for the chosen target (VEGF receptors). In the end, one must not forget that the process starting from drug discovery to the exploratory development to the full development of the drug is quite time consuming. But it is certainly not a deterring element towards such an approach.

Basic premise behind the activation of the drug: An enzymatic approach which involves the use of surface active enzyme coupled to an analogue/antibody will be most suitable in ensuring drug delivery (prodrug containing the desired copper complex to sequester oxygen at the endothelial-tissue interface) and its subsequent activation. The reason behind adopting such an approach is that a conjugate of this nature would not require internalization, thereby avoid any side effects associated. At the tumor site the prodrug will be activated by the conjugate (VEGF analogue/antibody bound enzyme). Some of the pre-requisites that might need attention here are that, firstly, the targeting moiety (analogue/antibody) and the bound activator (enzyme) must be compatible (of human origin) enough to avoid any immunological inactivation of the complex. Secondly, the activator shouldn't have any natural substrate in the region near the target cells so as to prevent any competition with the activation of the prodrug. Third, the enzyme activity of the conjugate should be not present or be present in minute levels; this is to circumvent any non-selective stimulation of the prodrug by Bredehorst. The activator coupled to the analogue can be an enzyme from any class; hydrolase, oxido-reductase, transferase, isomerase, lyase, or ligase by Napper. Reports by Imoto have suggested that the endoglycosidase lysozyme seems to be a suitable candidate for aforesaid. Activation of the prodrug by Bredehorst by the conjugate is accomplished by the usage of homo or

hetero bifunctional crosslinking agents or protein modifying agents which introduce highly reactive groups into the activator and the analogue moiety. Something similar can be achieved in the case of the hypothetical drug, designed to induce hypoxia in tumor cells.

The activation of the drug also depends on the presence of certain negatively charged residues which are conjugated to the complex (in this case VEGF analogue/antibody–enzyme) with the help of a cleavable spacer. This arrangement ensures that the presence of the negatively charged residue prevents the uptake of the prodrug by the tumor cells until the spacer is cleaved, later, enzymatically and the negatively charged residue is detached by Bredehorst. Hence, the hypothetical therapeutic drug, fortified with substantial experimental results from similar works, seems quite plausible. And thus an extensive research in the same may perhaps resolve things towards an inference.

Conclusion

If this notion works positively we are most likely to find cancer cells in a state of enhanced hypoxia that will be detrimental to their survival. This idea therefore will contribute, along with the multitude of other research works that are being practiced worldwide, in containing cancer. However, the efficacy of this approach remains elusive for now due to the lack of appropriate experimental certifications to support it. If not turned out well it should at least open up possibilities for a substantial research.

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