

Hypoxia or In Situ Normoxia: The Stem Cell Paradigm

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Although O_2 concentrations are considerably lowered in vivo, depending on the tissue and cell population in question (some cells need almost anoxic environment for their maintenance) the cell and tissue cultures are usually performed at atmospheric O_2 concentration (20–21%). As an instructive example, the relationship between stem cells and micro-environmental/culture oxygenation has been recapitulated. The basic principle of stem cell biology, “the generation-age hypothesis,” and hypoxic metabolic properties of stem cells are considered in the context of the oxygen-dependent evolution of life and its transposition to ontogenesis and development. A hypothesis relating the self-renewal with the anaerobic and hypoxic metabolic properties of stem cells and the actual O_2 availability is elaborated (“oxygen stem cell paradigm”). Many examples demonstrated that the cellular response is substantially different at atmospheric O_2 concentration when compared to lower O_2 concentrations which better approximate the physiologic situation. These lower O_2 concentrations, traditionally called “hypoxia” represent, in fact, an in situ normoxia, and should be used in experimentation to get an insight of the real cell/cytokine physiology. The revision of our knowledge on cell/cytokine physiology, which has been acquired ex vivo at non physiological atmospheric (20–21%) O_2 concentrations representing a hyperoxic state for most primate cells, has thus become imperious.

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Oxygen represents one of the most important factors influencing the evolution of life. Primitive life is considered to have started less than 4 billions years ago, and the first organisms that were capable to convert and store the energy of light in the form of biochemical molecules with higher energetic potential appeared less than 3 billions years ago. These organisms, by doing photosynthesis, started to release oxygen whose concentration in the atmosphere increased during the next billion years. About 2 billions years ago, the concentration of O_2 in atmosphere appeared as a factor influencing life. Oxygen was toxic for living organisms which developed several adaptation mechanisms aimed to detoxify it in parallel with the increase of O_2 concentrations in atmosphere which continued until 500 millions years ago. As a matter of fact, the eukaryots, whose physiology, based on the aerobic metabolism, is built during the evolution in order to detoxify O_2 , became dependent on it. However, O_2 concentration should be well adapted to particular cell metabolic type. In the human organisms, O_2 concentration varies significantly between the tissues: in the lung parenchyma and in circulation (McKinley and Butler, 1999; Saltzman et al., 2003; Johnson et al., 2005; Wild et al., 2005), as well as in well irrigated parenchymal organs (liver, kidneys, heart; Wölfle and Jungermann, 1985; Jungermann and Kietzmann, 1997; Roy et al., 2000; Welch et al., 2001; Mik et al., 2004) it is comprised between 14% and 4%. In other tissues, relatively less irrigated, O_2 concentration is even lower: in the brain, it varies from 0.5% to 7% (Whalen et al., 1970; Nwaigwe et al., 2000; Hemphill et al., 2005) in the eye (retina, corpus vitreous), from 1 to 5% (Buerk et al., 1993; reviewed in Yu and Cringle, 2005), in the bone marrow, from 0% to 4% (Tondevold et al., 1979; Chow et al., 2000).

Towards the Concept of “In Situ Normoxia”

In fact, in course of evolution, the cellular metabolism has been adapted to a moderate oxygenation, that is, oxygen concentrations that could be found in tissues (Massabau, 2000, 2003). This adaptation (reviewed by Kenneth and Rocha, 2008) initially concerns the modulation of molecular mechanisms existing in living organisms before the appearance of oxygen in atmosphere. For example, the stabilization of HIF-1 α transcripts, which originally initiated the synthesis of molecules acting in an anaerobic metabolism, shifted towards their

oxygen-dependent degradation. It is interesting to note that between anoxic condition and 5% of O_2 , the degree of stabilization of HIF-1 transcripts is inversely proportional to O_2 concentration (Jiang et al., 1996; Guzy and Schumacher, 2006). Over 5% O_2 , the HIF-1 transcripts are degraded. Nearly anoxic conditions result in an anaerobic metabolism, but at relatively higher O_2 concentrations, between 1% and 5% O_2 , the synthesis of highly energetic phosphates takes on thanks to a specific isoform of cytochrome c oxidase (Fukuda et al., 2007). For many cell populations these oxygen concentrations (1–5% O_2 ; O_2 concentrations existing in atmosphere approximately 1 billion years ago; Falkowski, 2006; Berner et al., 2007), called by Guzy and Schumacher (2006) “physiological hypoxia” are conditions of the physiological oxygenation, that is, the steady state oxygenation or “in situ normoxia.”

The adaptation induced by increased oxygenation of atmosphere conditioned the evolution of a complex circulatory and respiratory system to ensure an adequate O_2 supply for every cell type in organism. The new and very complex mechanisms such as cellular respiration (reviewed in Das, 2006) require O_2 as ultimate electron acceptor. These new mechanisms resulted in energy accumulation in the form of “high-energetic phosphates” that allowed a big progress in evolution enabling to build and maintain the organisms of complex structure and function, such as vertebrates. But the need for O_2 as a metabolic substrate is opposed to the risk of cellular macromolecules oxidative damage (ROS generation). That is why its concentration within cells is maintained within a narrow range that optimally balances supply and demand. A cell could and has to fight against the ROS in excess. This system,

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however, has its limits and oxygen concentration should not go beyond them to ensure the well being of cells (reviewed by Papandreou et al., 2005). Accordingly, it is obvious that the atmospheric O₂ concentration is too elevated for the cells of most tissues. Therefore it is evident that the basic condition to compare all other experimental conditions should represent the “in situ normoxia” for a certain cell type and not “atmospheric normoxia” considered, without any scientific justification, as a “standard tissue culture condition” and being, in fact, hyperoxia.

The “Mental Shortcuts”

Nevertheless, almost all present knowledge related to the action of cytokines and growth factors on cells and on the consequent cellular response (Metcalf, 2008) is based on the experiments at 20–21% O₂, that is, highly hyperoxic conditions. Apart practical reasons related to technical problems in controlling O₂ concentration, it is likely that negligence in the matter of ex vivo culture oxygenation originates from two paradigms: (1) Erythropoietin (Epo)-dependent regulation of Red Blood Cell (RBC) production and (2) Vascular Endothelial Growth Factor (VEGF) and its role in neovascularization. The first one is enabling the amplification of erythroid-responsive progenitor and precursor’s pool to increase the RBC production as a response to the tissue hypoxia resulting from a weak oxygenation of blood; the second one was usually assimilated to the neovascularization of hypoxic areas in tumors. Originally, the existence of a factor regulating erythropoiesis was postulated by Carnot and Deflandre (1906) and the Epo discovered (reviewed in Jelkmann, 1986) thanks to the observation related to high altitude-induced polycythemia. This phenomenon appears with decreased O₂ pressure (“high altitude hypoxia” or “hypobaric hypoxia”) comparing to sea-level O₂ one, which was then referenced as “normoxia.” As a consequence, the term “normoxia” for the atmospheric O₂ concentration was transposed, from classical physiology, to cell biology, that is, to ex vivo culture studies. Also, the term “hypoxia” was regularly associated with the pathology. Of course, these “mental shortcuts” neglect the facts that (a) a “normal” blood oxygenation is much lower than the atmospheric one (20–21% O₂) and it induces, however, a steady-state Epo production, ensuring the erythropoiesis in physiological conditions; (b) physiological tissue O₂ concentrations are even lower than arterial blood ones; (c) Epo acts as a physiological regulator in angiogenesis and brain development, that is, as a non-hematopoietic regulator (Arcasoy, 2008) (c) VEGF is acting as a key regulator of physiological angiogenesis during embryogenesis, endochondral bone formation, female reproductive functions (reviewed in Ferrara et al., 2003). . . In addition, the ex vivo experimentation mainly neglected the knowledge from in vivo physiology on the paradigm for oxygen-regulated gene expression (Stockmann and Fandrey, 2006) usually considering that the cytokines and growth factors other than Epo and VEGF are not concerned. Still, the list of genes coding cytokines, growth factor and their receptors, as well as other molecules exhibiting the “HIF-1-binding sequence”—hypoxia response element (HRE), is long (reviewed in Semenza, 2007) and increasing on a daily basis.

Oxygen Supply, Metabolic Feature, Proliferation and Differentiation: the Stem Cell Paradigm

Taken together, the metabolic/molecular biology studies performed on cell lines (Jiang et al., 1996; Guzy and Schumacher, 2006), show that moderately low O₂ concentrations (1–5% O₂) are permitting cell proliferation since providing the sufficient amount of energy by the adapted mitochondrial respiration. On

the contrary, in anoxic conditions, mitochondrial respiration has ceased and the energy is provided by an anaerobic glycolysis, thus insufficient to produce enough energy to support full cell proliferation and, especially, differentiation. An example from hematopoietic cell phenomenology is instructive: the IL-3 activates and stimulates the proliferation of CD34+ cells both at 3 and 20% O₂, but at very low O₂ concentrations approaching anoxia (0.1% O₂), CD34+ cells are inhibited in G0 or, if cycling, returned in G0 (Hermitte et al., 2006) irrespective of IL-3 presence. The action of different cytokine cocktails on stimulation on CD34+ cell proliferation was not prevented at 1–5% O₂ but the maintenance of stem cell activity is better at low O₂ concentrations than in air (Cipolleschi et al., 2000; Ivanovic et al., 2000a,b, 2002, 2004; Danet et al., 2003). The relationship between proliferation and differentiation rates is a parameter highly influenced by O₂ concentration, which remains to be elucidated. Nonetheless, it is evident that low O₂ concentrations (1–5%) decrease the differentiation rate of certain stem cell populations. In fact, it would be more appropriate to say “higher O₂ concentrations increase the differentiation rate” to be in line with the idea on the “negative evolution pressure” transposed to the “generation-age” hypothesis for heterogeneous hematopoietic stem/progenitor cell compartment (Rosendaal et al., 1979): the stem cells are highly sensitive to O₂, in order to be protected, its concentrations should be lower than 1% and approaching zero (Jang and Sharkis, 2007), that is, atmospheric O₂ concentrations existing between 2 and 3 billion years ago. By their metabolic properties (Jang and Sharkis, 2007; Simon and Keith, 2008) and their position in ontogenesis (Powers et al., 2008) stem cells reflect this early evolution stage (Massabau, 2000, 2003). In fact, if exposed to higher O₂ concentrations primitive stem cells “escape” death differentiating into more evolved forms, which are more adapted and resistant to higher O₂ concentrations (altogether is compatible with the general philosophy of Haeckel’s concept (Haeckel, 1866)). However this way the stem cells exhaust themselves. This scenario typically takes on at 20–21% O₂, that is, hyperoxic, atmospheric O₂ concentrations (Ivanovic et al., 2004).

We propose that self-renewing divisions of stem cells are conditioned by hypoxic metabolic type. Thus, a limited energy supply would allow only strict replication of DNA unlike activation of the transcriptional differentiation programs. So, the cell proliferates without the differentiation (a phenomenon called “self-renewal”). In fact, the low energy availability probably allows the expression of primary signaling pathways and transcription factors, which are, in general, highly evolutionary conserved (hence dating from very early evolution stages), which control stem cell self-renewal and multipotency (“stemness”), and are activated by HIFs, that is, by conditions typical for hypoxic metabolic cell type (reviewed in Keith and Simon, 2007 and in Lin et al., 2008). A stem cell could afford these “executive extensions” since their expression is compatible with a low oxygen metabolism; they ensure the cellular survival and/or basic cell reproduction functions but not the functions acquired at the ulterior evolutionary stages (“commitment”, “differentiation”, “specialisation”, “maturation”. . .). Typical examples are Oct 4 (Covello et al., 2006), Notch (Pear and Simon, 2005; Sansone et al., 2007) Wnt-signaling pathway (Lim et al., 2008), STATs (Wang et al., 2008), sonic hedgehog (Bijlsma et al., 2008), ATM-p38/MAPK (Bencokova et al., 2008). . . This situation is typically understood and considered as an “inhibition” of the differentiation. Of course, the principle is not “all or nothing” and, at different developmental stages and different oxygen availabilities, the stem cells could develop some aspects of aerobic (although still “hypoxic”) metabolism, permitting, to a different extent, the commitment and differentiation. Unlike the maintenance of primitive stem cells (usually CD34⁺ or

CD34^{low}), when their quiescent nature and slow proliferation are compatible with their low mitochondrial content (Freyer, 1998; Radley et al., 1999) and the hypoxic metabolic type (Parmar et al., 2007; the low energy demands), the differentiation events are associated with the mitochondria proliferation (“mitochondrial biogenesis”) which is paralleled by increase in aerobic metabolic properties of cells. So, the CD34⁺⁺⁺ and CD133⁺ cell populations which are, in fact, highly enriched in hematopoietic progenitors (very few of the cells characterized by this phenotype are the stem cells), are the richest in mitochondria (Piccoli et al., 2007; the authors wrongly consider CD34⁺ and CD133⁺ cells as “stem cells”). Indeed, the most recent data suggest a coupling between intrinsic metabolic parameters and stem cell fate (Schieke et al., 2008) and clearly show that repressing mitochondrial biogenesis and ROS by a molecular mechanism (TSC-mTOR), maintains quiescence and function (“stemness”) of hematopoietic stem cells (Chen et al., 2008).

The differentiation demands the internal and external signals that should be “cached” and “transmitted.” For example, to allow an efficient action of a cytokine, a cell should exhibit the receptors and operational corresponding signal transduction pathways. Thus, the effects of cytokines on stem and progenitor cells are depending on their metabolic state, which is crucial for the differentiation-related expression and synthesis (Mostafa et al., 2001b; Brunet de la Grange et al., 2006). Also, the response of cellular molecular machinery depends on the energy supply, thus on its metabolic type and actual O₂ concentration (Bell et al., 2007).

With respect to the stem cell fate, the concept presented above could be applied to two hypotheses: (1) the stem cells exhibit the same metabolic type as other cells, but, residing in highly hypoxic niches (Chow et al., 2000; Nilsson et al., 2001; Wilson and Trumpp, 2006; Arai and Suda, 2007; Parmar et al., 2007) they are quiescent (as proposed by Cipolleschi et al., 1993) or allowed to proliferate slowly without differentiation. This hypothesis is compatible with the concept of slow self-renewing divisions of somatic stem cells (Bradford et al., 1997); (2) stem cells exhibit a particular, peculiar metabolic type, permitting their proliferation without differentiation in highly hypoxic conditions, a property lost in course of differentiation. This concept would be compatible with the rapid self-renewing divisions of embryonnary stem cells and somatic stem cells ensuring maintenance of their pool during tissue regeneration; it is supported by the data on stem cell-specific metabolic features (Ito et al., 2006) as well as the one from thoughtful experiments of Dello Sbarba et al. (1987), showing that the stem cells proliferate in spite of respiratory chain saturation by piruvate that exhibits a cyostatic effect on progenitors. Finally, the stem cell population could be composed of both the stem cells exhibiting peculiar and “ordinary” metabolic type, adding a new dimension to the heterogeneity of the stem cell compartment.

Towards a Revision of Knowledge in Cell Biology

From the late seventies until today, a number of data have been accumulated clearly demonstrating that the cellular response (not only to Epo and VEGF) is depending on the actual O₂ concentration. For example, in the same culture medium and in presence of the same soluble factors, a completely different response of hematopoietic cells has been evidenced at low with respect to the atmospheric O₂ concentration (20–21%; Bradley et al., 1978; Broxmeyer et al., 1985, 1990; Smith and Broxmeyer, 1986; Koller et al., 1992a,b,c). Recently, we have demonstrated a positive effect of IL-6 on stem cell (pre-CFC) maintenance (and hence on the inhibition of stem cell differentiation) revealed only at 1 and not at 20% O₂ (Kovacevic-Filipovic et al., 2007). Basically the same

phenomenon was evidenced for VEGF (Brunet de la Grange et al., 2003). The real nature of stem cells, that is, their maintenance in G0 phase, self-renewal, commitment, etc., which are, of course, regulated by certain cytokines and growth factors, should be evaluated only at appropriately low O₂ concentrations characterizing the stem cell niche (Cipolleschi et al., 1993, 2000; Ivanovic et al., 2000a). Thus, here again, the response of stem cells to the cytokine stimulation is completely different at 0.1, 1, 3–5, or at 20–21% O₂, implying a physiological regulatory role of oxygen concentration in early hematopoiesis (Ivanovic et al., 2000a, 2002, 2004; Hermitte et al., 2006). The response of committed progenitors to cytokine stimulation concerns all hematopoietic lineages (Laluppa et al., 1998; Mostafa et al., 2000, 2001a,b). It was pointed for example that “O₂ tension alters the effect of cytokines on the megakaryocyte, erythrocyte, and granulocyte lineage” (Laiuppa et al., 1998). This point is being completely confirmed in the ex vivo model of erythropoiesis: an appropriately low O₂ concentration is required for each developmental stage of erythropoiesis to optimize the ex vivo production of RBC from CD34⁺ cells (Vlaski et al., 2007). With that respect, the O₂ gradient seems to be a general physiologic regulator, beyond well-known, Epo-related downstream tuning (Cipolleschi et al., 1997; Vlaski et al., 2007; submitted). Recently, a series of papers studying different cell types confirm that the actual O₂ concentration determines the cellular response to cytokines as well as cytokine secretion pattern by the cells (Carswell et al., 2000; Morrison et al., 2000; Csete et al., 2001; Lennon et al., 2001; Desplat et al., 2002; Arnett et al., 2003; Fink et al., 2004; Moussavi-Harami et al., 2004; Scherer et al., 2004; Grinakovskaya et al., 2007; Motohira et al., 2007; Schutyser et al., 2007; Wang and Wang, 2007; Battaglia et al., 2008; Kanichai et al., 2008; Kim et al., 2008; Mancino et al., 2008; Ricciardi et al., 2008). In addition, the DNA repair seems to operate better at low O₂ concentration (Nijnik et al., 2007).

Concluding Remarks

In order to stress the importance of oxygenation for stem cell fate, the “oxygen stem cell paradigm” was elaborated integrating the “generation-age hypothesis” and hypoxic metabolic properties of stem cells with the oxygen-dependent evolution of life and the position of heterogeneous stem cell populations in ontogenesis, (in line with the general frame of Haeckel’s concept). With that respect, stem cells reflect an early evolutionary stage characterized with very limited O₂ availabilities, that is, reminding those unicellular organisms still not completely adapted to oxygen, exhibiting the “hypoxic” metabolic type. So, the self-renewing divisions of stem cells are conditioned by hypoxic metabolic type since a limited energy supply would only allow (i) the strict replication of DNA unlike activation of the transcriptional differentiation programs, and (ii) the expression of highly evolutionary conserved (hence dating from very early evolution stages) signaling pathways and transcription factors involved in control of stem cell self-renewal and multipotency (“stemness”), in other words only enabling the cell survival and proliferation but not commitment and/or differentiation.

The O₂ supply is finely regulated in vivo, with respect to the cell and tissue type. The tissue O₂ concentrations are much lower than the atmospheric ones. Since the cellular response is dependent on O₂ concentration, to get an insight of the real cell physiology, it should be studied only at appropriate O₂ concentrations approximating at the best in situ normoxia for each cell type. With that respect, the atmospheric O₂ concentration could not be considered as “normoxia.”

This argumentation urges the revision of our knowledge on cytokine biology, which is acquired ex vivo at non physiological atmospheric (20–21%) O₂ concentrations representing a

hyperoxic state for most primate cells, except mature cells originating from the tissues which are in direct contact with air (such as skin surface, mouth and respiratory epithelium. . .).

The low oxygen, undoubtedly, better approximates the in vivo environment.

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