Histol Histopathol (2007) 22: 85-90

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Protection from oxidative stress by enhanced glycolysis; a possible mechanism of cellular immortalization

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Summary. Reactive oxygen species (ROS) play a crucial role not only in the physiological signal transduction but also in the pathogenesis of several human diseases such as atherosclerosis, neuro-degenerative diseases, metabolic disorders, aging or cancer amongst others. Oxidative stress is also responsible for cellular and organism senescence, in accordance with what Harman initially proposed in the free radical theory of aging. Recent findings support the notion that protection from oxidative stress can increase life span significantly. We reported that enhanced glycolysis could modulate cellular life span with reduction of oxidative stress. Moreover, the tumor suppressor gene p53 controls post-transcriptionally the level of the glycolytic enzyme, phosphoglycerate mutase (PGM). As enhanced glycolysis is a distinctive and prominent feature of cancer cells (termed the Warburg effect), our findings disclosed a novel aspect of the Warburg effect: the connection between senescence and oxidative stress.

Key words: Glycolysis, Senescence, the Wardburg effect, Phosphoglycerate mutase (PGM), Oxidative stress

Introduction

There are several hallmarks that distinguish cancer cells from their normal counterparts, such as unlimited replicative potential, growth factor independence, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000). Enhanced glycolysis is also a well-known characteristic of cancer cells. This property is well utilized in clinical practice for the detection of metastatic tumor mass by positron-emission scanning of 2-[^{18}F]fluoro-2-deoxy-D-glucose (Gambhir, 2002).

A high glycolytic rate, even under high oxygen conditions, is referred to as the Warburg effect. Dr. Otto Warburg first reported that cancerous tissues or cells display increased glycolysis by an unknown mechanism (Warburg, 1930). It was widely assumed that cancer cells maintain upregulated glycolytic metabolism to adapt to the hypoxic condition *in vivo*, as solid tumors overgrow the blood supply of the feeding vasculatures. Alternatively, it has been proposed that the increased glucose flux might improve efficiency of glucose utilisation in a microenvironment in which glucose is limited. In such a context, the glycolytic response represents a successful metabolic adaptation of cancer cells *in vivo* (Gatenby and Gawlinski, 2003). However, these do not explain the constitutive metabolic change that maintains high glycolytic rates in cancer cells even in the presence of 20% oxygen in vitro. Thus, although the observation of the Warburg effect is well established, its detailed molecular mechanism remains to be clarified. The concomitant induction of angiogenesis and glycolysis with cell proliferation is mediated partly by activating hypoxia-inducible transcriptional factor (HIF-1). Hypoxia increases HIF-1 levels in most cell types and HIF-1 mediates adaptative responses to changes in tissue oxygenation. Thus, HIF-1 can directly upregulate expression of a set of genes involved in both local and global reaction to hypoxia, including angiogenesis, erythropoiesis, breathing and most of the glycolytic enzymes: HK1, HK2, AMF/GPI, ENO1, GLUT1, GADPH, LDHA, PFKBF3, PFKL, PGK1, PKM, TPI (Semenza, 1998). Interestingly, both HIF-1 and glycolytic enzymes are overexpressed in many tumors and cancer cells (Dang and Semenza, 1999). Altogether

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these data support a functional link between enhanced glycolysis and cellular adaptation during tumor formation and expansion.

Recently, we reported that enhanced glycolysis can bypass cellular senescence, while inhibition of glycolysis leads to premature senescence. Moreover, this effect was found to be associated with oxidative stress regulation (Kondoh et al., 2005b). These findings might uncover an unexpected role of the Warburg effect in cellular immortalization, which we propose as a critical and early step of tumorigenesis. This review spotlights novel aspects of the Warburg effect related with senescence and oxidative stress.

Senescence induced by oxidative stress

Most somatic cells have a limited replicative potential under standard tissue culture conditions and suffer permanent cell cycle arrest, called replicative senescence (Hayflick and Moorhead, 1961). Senescent cells display distinct properties, such as enlarged and flattened appearance with single prominent nuclei, increased expression of PAI-1, and senescence associated ß-galactosidase activity among others features (Campisi, 2001). Although these cells are metabolically viable, they show negligible DNA synthesis, as judged by BrdU incorporation.

In human primary fibroblasts, one of the principal senescence triggers is the loss of telomeric repeat sequences at chromosome ends as a result of cell division. Severe telomeric erosion during serial passages provokes a permanent cell cycle arrest in tissue culture. A specific RNA reverse transcriptase called telomerase extends telomeres and its ectopic expression can restore the proliferative capacity of primary human fibroblast. Interestingly, although mouse telomeres are much longer than their human counterparts (60 Kb versus 12 Kb), mouse embryonic fibroblasts (MEFs) stop growing very rapidly compared to human primary fibroblasts. Besides telomere structure, there are several other factors influencing cellular life span, as some cell types reach a proliferative barrier long before telomere erosion has become crucial (Wright and Shay, 2002). It is now admitted that cells can arrest virtually at any age, in a telomere-independent manner (Sherr and DePinho, 2000). For example, oncogenic-stimuli can induce premature senescence, called oncogene induced senescence (Serrano et al., 1997; Collado et al., 2005). This arrest is phenotypically similar to those of replicative senescence. Thus, cellular senescence also constitutes a potent anticancer mechanism (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005).

Recent studies suggest that the accumulation of ROS and oxidative damage (Lee et al., 1999; Parrinello et al., 2003; Kondoh et al., 2005b), are involved in senescence occurrence. ROS, such as superoxide anion and hydroxyl radical, are produced during cellular metabolism mainly in the mitochondria. ROS are also produced in response to different environmental stimuli such as UV, IR, chemicals, hyperoxia or hydrogen peroxide treatment (Beckman and Ames, 1998). Normal levels of cellular ROS can work as second messengers involved in several signal transduction pathways (such as the Ras-Raf-MEK cascade) (Irani et al., 1997). But abnormal ROS accumulation and its side-effects over intracellular macromolecules (oxidation of lipid, protein and DNA) provokes cumulative damage at cell, tissue, and organism level.

In a normal environment, approximately 90% of the oxygen that enters into the cell is used to produce energy via the mitochondrial respiratory chain. During this process, four electrons are added to each O_2 molecule together with $2H^+$, resulting in the liberation of two molecules of water. It is estimated that 1-4% of the O_2 uptake into cells forms partially reduced oxygen species or ROS (Barzilai et al., 2002). Since the initial hypothesis of the free radical theory of aging by Harman (Harman, 1956), a close link between organism aging and oxidative stress has been suggested (Dillin et al., 2002; Larsen and Clarke, 2002; Lee et al., 2003; Rea and Johnson, 2003). Recent studies suggest that oxidative stress can also have an impact on cellular life span in tissue culture (Fig. 1). Mild oxidative stress (for example, treatment with low concentrations of hydrogen peroxide) is enough to induce senescence (Chen et al., 1995, 2001). Interestingly, premature senescence induced by culture-stress or oncogene-induced stress is associated with oxidative damage in cells.

Noteworthy, increased ROS accumulation is also observed during replicative senescence. The replicative potential of both murine and human fibroblasts are

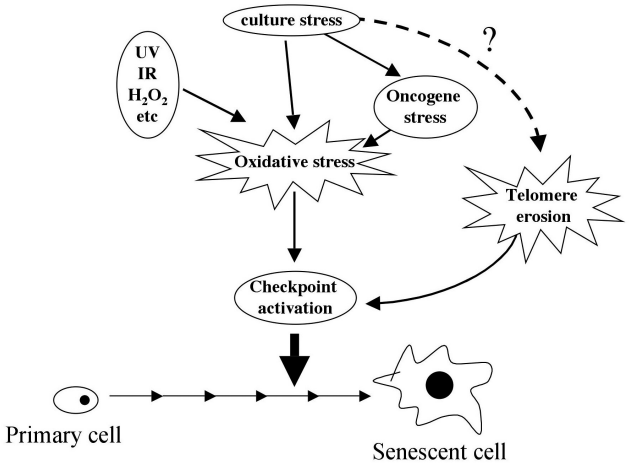

Fig. 1. Oxidative stress induces telomere-independent senescence. Senescence is induced in a telomere-dependent or -independent manner. Ectopic expression of telomerase can bypass replicative senescence. Several stimuli can induce oxidative stress. Accumulation of oxidative damage provokes premature senescence, which could be overcome by ectopic expression of glycolytic enzymes. See text for details.

significantly extended under low oxygen, associated with less oxidative damage inflicted than under normoxia (O_2 20%) (Itahana et al., 2003; Parrinello et al., 2003). Immortalised cells suffer less oxidative damage than primary fibroblast when cultured under 20% O_2 (Kondoh et al., 2005b). Moreover immortalized cells are more resistant to the deleterious effects of hydrogen peroxide than primary cells (Kondoh et al., 2005b; Lee et al., 2005). Thus, ability to resist oxidative stress could be a clue for explaining the immortality of cancer cells. To elucidate how immortal cells are protected from oxidative stress, it is important to understand how normal cells try to adapt to the oxidative stress that they are subjected to during senescence (Kondoh et al., 2005b).

Protection against oxidative stress prevents senescence

Normal cells try to efficiently remove ROS in order to avoid the deleterious effects of oxidative stress. To this end, several enzymes scavenge the ROS produced in the cells (Fig. 2). The superoxide dismutase enzyme (SOD) converts superoxide anions into hydrogen peroxide. Hydrogen peroxide can be detoxified by catalase, glutathione peroxidase (GP), or peroxiredoxin (PRX) into water (Barzilai et al., 2002). The reaction catalyzed by TRX or GP requires other cellular dithiol proteins such as thioredoxin (TRX) or reduced glutathione (GSH) respectively. They are kept in their reduced forms through the reducing force of NADPH.

Consequently, these antioxidant enzymes can impact both on the proliferation of primary and immortal cells as they should counteract ROS effects. The ability of SOD to bypass senescence has been well studied and established in various cells or organisms. Increased expression of SOD can extend the life span of primary fibroblasts (Serra et al., 2003). Conversely, knockdown of SOD using siRNA induces premature senescence accompanied by p53 activation (Blander et al., 2003). Transgenic fly overexpressing SOD (Parkes et al., 1998) or the detoxifying enzyme catalase (Orr and Sohal, 1994) presents an extended organism life span. Treatment of cells with the pro-oxidant agent menadione leads to elevation of GSH, a strong apoptotic response and reduced clonogenic survival (Coe et al., 2002). Specific inhibition of gamma glutamylcysteine synthetase (γGCS) results in the depletion of the GSH pool and prevents menadione-induced increase in GSH, sensitizing cells to oxidative stress. TRX might also have an anti-ageing role as TRX-expressing transgenic mice displayed a moderate but significantly extended life span (Mitsui et al., 2002).

Although it is clearly established that these anti-oxidant scavengers are essential for proliferation of immortal cells (Castro-Obregon and Covarrubias, 1996), little is known so far on the specific regulation operating in cancer cells. It might be worth studying these regulatory mechanisms, as increasing evidence suggest their critical role in cellular senescence and organism ageing. Interestingly, the anti-oxidant function of some scavengers (such as GSH or TRX) is closely coupled to the NADPH/NADP balance. Most of the NADPH/NADP are produced through the pentose phosphate pathway (PPP), a branching metabolic pathway derived from the glycolytic pathway (Fig. 2). Our recent findings linking glycolysis and senescence might give a clue to explain how immortal cells maintain their anti-oxidant scavenger capacity.

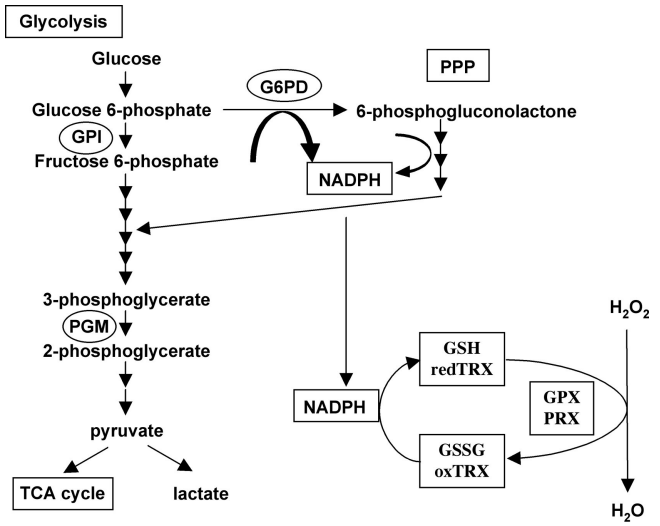

Fig. 2. Glycolysis and pentose phosphate pathway (PPP). Circled enzymes are reported to be involved in cellular senescence. NADPH is provided via PPP as the reducing force. GSH: reduced gluthation, redTRX: reduced thioredoxin, GSSG: oxidized gluthation, oxTRX: oxidized thioredoxin, GPX: glutathion peroxidase, PRX: peroxiredoxin.

Senescence-bypassing effect of enhanced glycolysis

We recently found that glycolytic enzymes can modulate cellular life span of MEFs (Kondoh et al., 2005b). In a senescence bypassing screening in MEFs using a retroviral cDNA library, we isolated the glycolytic enzyme phosphoglycerate mutase (PGM). PGM converts 3-phosphoglycerate to 2-phospho-glycerate during glycolysis (Fig. 4). Analysis of the impact of other glycolytic enzymes over senescence in MEFs showed that glucophosphate isomerase (GPI) could also drive immortalization of MEFs. Ectopic expression of PGM or GPI increases glycolytic flux, decreases the oxidative damage that MEFs are exposed to, and extends the life span of primary MEFs. Conversely, knockdown of PGM or GPI via specific siRNA induces premature senescence. Moreover, we and others found that the glycolytic flux declines during senescence both in murine and human fibroblasts (Zwerschke et al., 2003; Kondoh et al., 2005b).

How can an increase in glycolysis immortalize primary cells? From the data discussed above it seems

that enhanced glycolysis can protect cells from oxidative stress and as a consequence avoid senescence (Kondoh et al., 2005b). MEFs immortalized by PGM or GPI suffer less oxidative damage than control cells as estimated by cytosolic ROS staining, or quantification of 8-hydroxydeoxyguanosine (8-OHdG), a hallmark of oxidative DNA damage lesions.

Interestingly, mouse ES cells present a surprisingly high glycolytic rate (Kondoh et al., 2005a). Thus similarly to cancer cells, ES cells display the Warburg effect. We hypothesized that reversible modifications such as a specific factors playing a role in ES cells immortality enhances the glycolytic rate resulting in an increased protection from oxidative stress (Kondoh and Gil, 2006). This metabolic protection might contribute to preserve the genome integrity of ES cells avoiding genetic alterations and allow them to maintain their self-renewal capacity. Also, these metabolic rates can be reverted, which would explain why differentiated cells do not present this enhanced metabolic levels.

Although this remains a hypothesis, several reports support it. The impact of glucose-6-phosphate dehydrogenase (G6PD) activity on cell proliferation is well established (Tian et al., 1998). G6PD catalyzes the rate-limiting step in the pentose phosphate pathway (PPP), which is responsible for the recycling of NAPDH and maintenance of the redox balance as described above. G6PD-deficient human fibroblasts have a reduced lifespan that is attributable to oxidative stress and can be corrected by the ectopic expression of this enzyme (Cheng et al., 2004; Ho et al., 2000). Both G6PD activity and the NADPH pool decline during continued culture passage, presumably as a consequence of the accumulation of oxidative damage (Cheng et al., 2004). Importantly, ES cells ablated from G6PD expression are extremely sensitive to oxidative damage, showing massive apoptosis at low concentration of oxidants that are not lethal for wild type ES cells (Filosa et al., 2003). It would, therefore, be worth exploring whether enhanced glycolysis can then promote increased NADPH production via the PPP and exert its anti-senescence function.

Molecular regulation of glycolysis

An increase in glycolysis is one of the hallmarks of most cancers, as first noted by Otto Warburg over seven decades ago (Warburg, 1930). However, the Warburg effect cannot be simply explained by the cellular adaptation to hypoxia for several reasons. First, under standard culture condition (20% oxygen), cancer cells still show enhanced glycolysis, which suggests stable alterations at the genetic or epigenetic level (Gatenby and Gillies, 2004). Second, the ectopic expression of HIF-1 causes cell cycle arrest, implicating that HIF-1 overexpression is not enough for the advantageous growth of cancer cells. Third, anaerobic glycolysis is metabolically inefficient, producing only two moles of ATP per mole of glucose, compared to mitochondrial respiration, 36 moles of ATP per mole of glucose. We reported that enhanced glycolysis can render primary cells resistant to senescence effect by diminishing oxidative stress. Our findings also suggest that enhanced glycolysis can be an early event in tumorigenesis, necessary to overcome the senescence barrier of primary normal tissue. Thus, the novel facets of the Warburg effect are exposed with these recent investigations.

Finally, it is worth exploring the detailed molecular regulatory mechanism of glycolysis, as modulation of glycolysis can have broad physiological effects (Fig. 3). For example, several glycolytic inhibitors are under investigation as anti-cancer drugs (Garber, 2004). Different oncogenes (e.g. ras, cmyc, src) or signaling kinase (Akt, AMPK etc) can regulate glycolysis (Carling, 2004; Elstrom et al., 2004). Moreover, recent work indicates that the tumor suppressor p53 is involved in the regulation of glycolytic metabolism (Dang and Semenza, 1999; Kondoh et al., 2005b). One possible interpretation could be that one of the major targets of p53 involved in senescence induction impacts on metabolic regulation, which render cells sensitive to oxidative stress. Identifying this factor would provide further links between oxidative stress and cellular life span.

HIF-1 transcriptionally regulates most of glycolytic genes with the only exception of PGM (Iyer et al., 1998; Niizeki et al., 2002). We reported that the p53 status can affect PGM protein level post-transcriptionally. PGM must be a target of anti-cancer drugs in specific types of cancer as PGM might be the rate-limiting enzyme in some specific cells (Xu et al., 2005). PFK has long been believed to act as the rate-limiting enzyme for glycolysis, but its ectopic expression has a minor impact on the glycolytic flux in immortal and primary cells (Urbano et al., 2000; Kondoh et al., 2005b). Recent findings suggest that the regulation of glycolysis

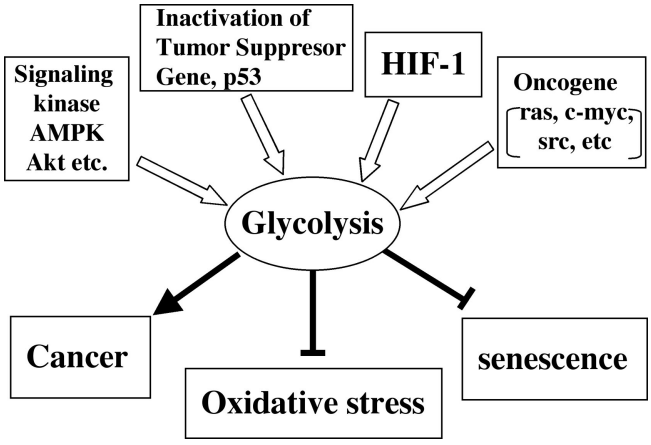

Fig. 3. The molecular regulation of glycolysis. Glycolysis does not only serve as a source of ATP, but also has several profound physiological effects. Key molecules for glycolytic regulation are described.

depends on cellular context. As many regulatory molecules for glycolysis are being identified, its complexity and interplay will be a matter for future studies.

Acknowledgements. Jesus Gil is funded by the MRC in UK.

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Accepted May 31, 2006