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The Nuclear Compartmentation of Glutathione: Effect on Cell Cycle Progression

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1. Introduction

The oxidizing environment, shared by all aerobic organisms and crucial for their survival, poses a continuous threat to cellular structures all the living beings are made of. Structural proteins and lipids, and cellular membranes they compose, nucleic acids and enzymes that govern vital cellular processes are all susceptible to oxidative damage. Dealing with this inevitable and constant danger is one of the greatest challenges the living being with aerobic metabolism has to meet.

2. The implication of cellular redox balance in cell proliferation

2.1 Reactive oxidative species in cell proliferation

Classical work of Kelvin Davies (Davies, 1999), more than ten years ago, showed that the cells show a whole range of responses to oxidative stress that depends on the intensity of the stress. Low level of hydrogen peroxide induced mitogenic responses and stimulation of proliferation; this observation was firstly reported by Oberly (Oberly et al., 1981) who have described that oxidative stimuli, such as superoxide and hydrogen peroxide, could activate signalling pathways that lead to proliferation. Davies et al. further assert that considerable increase in the oxidant concentrations caused temporary growth arrest which became permanent with a progressive increase. When high H_2O_2 concentrations were used, apoptosis took place and at very high oxidant levels the cells were killed by necrosis. A year later, (Pani et al., 2000) demonstrate a causal link between redox changes and growth control by cell density: they show that low level of oxygen species in the environment of proliferating cells was not only stimulating but necessary for the correct mitogenic signaling. This study was immediately followed by the work of Menon et al., 2003 who suggested that an oxidation event early in G1 phase may be a critical regulatory step in the progression of the cells into S phase. This lead to the development of the model of the "redox cycle within a cell cycle" proposed by the same group several years later (Menon & Goswami 2007).

According to this model, the transient change in ROS could modify the redox state of cell cycle regulatory proteins, at their critical cysteine residues, and thus determine progression or arrest in the proliferation. Antioxidant mechanism could scavenge ROS and reverse the process. In accordance to these reports, Barry Halliwell (Halliwell, 2007) draw a complete

view of the present knowledge of the role of oxidative stress in promoting cancer, its damaging effects to DNA, and its action on cell proliferation and apoptosis. Malignant cells produce more radical species and, although antioxidant defence could also be induced in these cells, they display a pro-oxidant state. However, apparently the oxidative stress generated in these high proliferative cells does not exceed the level where oxidative damage becomes so severe that cell function is impaired. This finding is in line with previously cited reports and many others that support the role of reactive oxidative species mediated signalling in the promotion of cell growth.

2.2 The bridge between the oxidative stress and cell proliferation - Glutathione

Glutathione (GSH) is the most abundant non-protein thiol in mammalian cells (Meister & Anderson, 1983). It is considered essential for survival in mammalian cells (Viña, 1990) and yeast Meister & Anderson, 1983; Viña et al., 1978), but not in prokaryotic cells. The exact nature of this important difference has not been elucidated. Glutathione was discovered in 1888 by Rey Pailhade as "organic hydrogenate of sulphur" (Rey Paihade, 1988) and "rediscovered" and fully described by Sir Frederic Gowland Hopkins in the 1920s (Hopkins, 1929) and quoted by Sies several years later (Sies, 1999).

Glutathione has attracted the scientific interest with variable intensity along the century since its discovery and many important cellular functions of this tripeptide were revealed along the years. Glutathione shows a widespread localization within cells and considerably high concentration in cells and tissues (up to 10 mM) (Tateishi et al., 1974). Examples of normal physiological functions of glutathione known for a long time include regulation of the transport of certain amino acids (Viña & Viña, 1983) control of cytoskeleton assembly (Burchil et al. 1978) and regulation of enzymatic activity (Ernst et al., 1978; Ziegler, 1985). During 1960s, GSH was demonstrated to be a co-substrate for a number of important enzymatic reactions: GSH-S-transferase was described (Booth, 1961) and its role in a first–line defence against electrophilic insult, obviously dependent on glutathione, was suggested (Boyland, 1969). These pioneer works became the bases for many studies that lead to the development of concepts such as drug and foreign compound detoxification, and multidrug resistance (Smith, 1977) of crucial importance in the modern cancer therapy. Glutathione, as it lacks toxicity linked to cysteine (Viña et al., 1983), is considered perfect as a cellular thiol "redox buffer" with a purpose to maintain a given thiol/disulfide redox potential (Sies, 1999). Therefore, the redox properties and abundance that characterize this molecule grant it a major role in protecting the cell against oxidants and electrophiles, and during 1980s this particular role of glutathione is central in many research efforts.

Association of redox regulation with toxicity events lead to the introduction of the concept of "oxidative stress" at biochemical and cellular level (Sies & Cadenas, 1985). Oxidative stress is generally defined as an imbalance between prooxidants and antioxidants with a considerable effect on other cellular components, including redox sensitive functional groups of proteins. Nowadays, with the increasing awareness of the importance of ROS and glutathione in cellular signalling, and the cellular redox environment in fundamental physiological processes, a new definition of oxidative stress is proposed. According to Jones, 2006, oxidative stress may be better defined as a disruption of redox signaling and control. Interestingly, more than 10 years ago, searching for a molecular link between oxidative stress and cell proliferation, Cotgrave IA and Gerdes RG recommended similar term: "oxidant mediated regulation" (Cortgreave and Gerdes, 1998).

2.3 Glutathione in cell proliferation

Several studies from more than 20 years ago have suggested that changes in low molecular weight thiols (LMWT) are associated with regulation of cell growth. Harris and Patt published (Harris & Pat, 1969) that nonproliferating mouse tumour cells contained LMWT than proliferating cells and in early eighties various authors report similar results: human lung and ovarian tumour cells during the exponential growth demonstrate higher GSH levels than during nondividing state (Harris & Pat, 1969; Post, 1983). In accordance to these findings, Kosower and Kosower (Kosower & Kosower, 1978) have demonstrated that decrease of GSH biosynthesis in vivo inhibits tumour growth rate. Moreover, it was suggested that cellular GSH may have to reach certain critical levels before proliferation can be initiated and that variations in the protein sulphydryl redox status may directly relate to regulation of cell growth (Atzori et al. 1990).

Defining the intrinsic cellular redox environment by estimation of glutathione (GSH)/glutathione disulfide (GSSG) redox state, the group of Dean P. Jones (Nkabyo et al. 2002) concluded that each phase in the life of the cell is characterized by the certain redox state. Proliferating cells are in the most reduced state, with the values of Eh between -260mV and -230mV (Schafer & Buettner, 2001). Upon a growth arrest caused by differentiation (Nkabyo et al. 2002) or contact inhibition (Schafer & Buettner, 2001) cells are 40 mV more oxidised (-220mV to -190mV) while the apoptotic process is accompanied by further oxidation up to -165mV (Sun & Oberley, 1996).

Therefore, while the cell progresses from proliferation, through contact inhibition, differentiation, and finally apoptosis, there is an intrinsic and natural progression from more reduced to more oxidised cellular redox environment. The universality of this model which applies to various cells from different organisms (reviewed in Schafer & Buettner, 2001) inspired a daring hypothesis of Schafer and Buettner on the implication and function of thiols and disulfides as nano-switches. The GSSG/2GSH couple is imagined as a switchboard that move the cell from proliferation through differentiation towards programmed cell death, if the redox environment could not be maintained, or necrosis when the oxidative insult is to severe.

2.4 Glutathiolation of regulatory proteins as a link between a stimulating oxidative event and reduced cell environment in cell proliferation

During the last two decades the increasing body of evidence reveals that several transcription factors undergo oxidant modification necessary for their activation. For instance, the property of binding DNA and thus regulate gene expression of AP1, NfkB, p53, and SP1 depends on the redox status of cysteinyl thiols in their structures (Sun & Oberley, 1996). Thus, the idea of protein glutathiolation as a regulatory mechanism of importance in cell proliferation came into sight.

Glutathiolation is a protein modification which consists in the covalent union of the tripeptide glutathione to the SH group of the cysteine residue. For a long time this reaction was considered to be a consequence of the equilibrium between protein thiols and GSSG inevitably related to oxidative stress. From this point of view, glutathiolation fulfills two important functions. Firstly, its reversibility enables the preservation of glutathione in the cell and serves as a buffer for the reduction potential; otherwise, GSSG efflux would cause the loss of GSH from the cell, decreasing the reducing capacity which could be recovered only by the synthesis of new GSH (Schafer & Buettner, 2001). Secondly, it provides

protection for protein-SH against irreversible modifications and protein damage in response to higher levels of oxidative stress (Dalle-Donne et al., 2007). Interestingly, it was demonstrated that glutathiolation as a posttranslational modification occurs not only during oxidative stress, but also under basal conditions and is involved in regulating distinct transcription factors, such as NF-kB (Pineda-Molina et al., 2001), its inhibitor factor IKK (Reynaert et al., 2006) and c-Jun (Canela et al. 2007). Apparently, the binding capacity of these proteins to DNA or other proteins is modulated by glutathiolation. This relatively recent focus on the implication of glutathiolation modulatory effects on protein function yielded important breakthrough in elucidation of the implication of this modification in various physiological and pathological situations (Giustarini et al. 2004) and raises interesting questions about its possible implications in cell proliferation.

2.4.1 The role of glutathione in DNA synthesis

Among the important roles that GSH plays in cellular physiology, and among the first to be described, was its role in DNA synthesis. The pentose phosphate pathway is a cellular source of NADPH that is involved in reductive biosynthesis. In this process, ribose-5-phosphate is formed and subsequently used for the synthesis of RNA, DNA and nucleotide coenzymes. Apart from the synthesis of nucleotides, NADPH is also required for the formation of amino acids, fatty acids, cholesterol, neurotransmitters and nitric oxide (NO). Furthermore, NADPH is the source of electrons in the process of reduction of ribonucleotides to deoxyribonucleotides catalyzed by the ribonucleotide reductase (Thelander & Reichard, 1979).

The route is initialized by two distinct but complemented systems, the thioredoxin system and the glutaredoxin system. Thioredoxin operates by transferring the electrons to ribonucleotide reductase, and they are supplied by the thioredoxin reductase and NADPH. The glutaredoxin system is initialized by the glutathione reductase, which reduces the GSSG to GSH using the NADPH as source of electrons. GSH is used by the glutaredoxin to provide the reducing power to the ribonucleotide reductase (Zahedi et al., 2009).

The crucial role of glutathione in DNA synthesis has been extensively documented (Thelander & Reichard, 1979; Holmgren, 1976). For instance, Dethlefsen and co-workers (Dethlefsen et al. 1988) showed that glutathione depletion inhibits DNA synthesis in mammary carcinoma cells. In addition, the vital importance of GSH in this process has also been demonstrated in human T lymphocytes (Suthanthiran et al., 1990). As mentioned above GSH is an indispensable requirement in eukaryotes. In contrast, it does not demonstrate the same importance in prokariotes. It has been shown that E. Coli lacking gshA, the rate limiting enzyme in the synthesis of GSH, can grow without GSH supplementation (Greenberg & Demple, 1986; Miranda-Vizuete et al., 1996). On the contrary, in yeast, the depletion of GSH does affect cell proliferation on the level different from DNA synthesis: mutants deficient in GCS after GSH withdrawal arrest cells in G1, whereas a strain with a defect in ribonucleotide reduction arrest cells in S phase (Wang et al., 1997). Since other possible explanations, such as protection against oxidative stress or protection against non-native protein disulfides, have been discarded (Spector el al., 2001), it appears that the essential function of GSH in yeast is related to the redox properties of its thiol group. Consequently, glutathione can be replaced by dithiothreitol, but not with a GSH analog where a thiol group has been substituted by a methyl group (Grant et al., 1996). Since GSH is the reductant for glutaredoxin as explained previously, and glutaredoxin is also

essential (Rodriguez-Manzaneque et al., 1999) presumably their vital importance may be interdependent. Then GSH seems to be important in S phase. During the process of DNA replication, errors, such as double-strand breaks (DSBs) that arise from stalled replication forks, require attention by the DNA damage response proteins. Thus, the correct control of DNA synthesis and probably essential molecules, such as GSH, are necessary for the correct DNA processing.
One of the most important proteins involved in DNA damage signaling pathway is the ataxia-telangiectasa mutated protein (ATM). This central signaling protein, mainly for DSBs, is involved in the repairing DNA process necessary after replication stress. Thus cells lacking ATM fail to execute many of the cellular responses to DNA damage (Zhou & Elledge, 2000). In addition, control of ATM responses after DNA replication may be necessary for the correct cell cycle control. In that way, ATM is a central component in the cell cycle regulation. Therefore, patients with ataxia telangiectasia have reduction in DNA synthesis (Painter & Young, 1980). Furthermore, a recent work published by Guo Z. and coworkers describes using a series of elegant experiments how ATM sense the redox changes to modulate their activity (Guo et al, 2010). Interestingly, these authors propose that ATM may regulate global cellular responses to oxidative stress, remarking the essential link between redox control and DNA interacting, remodeling or repairing proteins. In Fanconi anemia for instance, Castillo and coworkers have shown that ATM dependent phosphorylation of FANCD2, one of the main proteins in the Fanconi anemia pathway of DNA repair, is necessary for normal S-phase checkpoint activation after oxidative stress (Castillo et al, 2011).

2.4.2 Regulation of telomerase activity by glutathione

The eukaryotic chromosomes are capped by telomeres, which consist of TTAGGG DNA sequences repeated in tandem, associated with several proteins, which protect the final regions of chromosomes. These structures play an important role in the stability and the complete replication of the chromosomes. Conventional DNA polymerases cannot fully replicate the 3′-end of the lagging strand of linear molecules, and therefore in every cell division telomeric sequences are lost (Komberg, 1969). Telomerase is an important enzyme that ensures the maintenance of normal telomere length. This activity is high in human cancers (Kim et al., 1994), but virtually absent in normal human tissues, except germinal cells (Harley et al., 1990). Telomerase regulation is not completely understood, but its changes are related to both cancer and aging (Sharpless & Depinho, 2004). Studies carried out by Jady et al. show that human telomeres are more accessible during the S-phase (Jady et al., 2006) and that the telomerase assembly with telomeres takes place at this specific moment of the cell cycle (Jady et al., 2006; Tomlison et al., 2006). Telomerase plays a key role in cellular homeostasis, because it maintains the length of the telomeres. This especially important in germinal cells in which it is necessary to keep a normal telomeric length after many cellular divisions. Important contributions about the epigenetic control of telomeres have been reported recently (Koziel et al., 2011). In that way, Maria Blasco has suggested that telomeres are under epigenetic control (García-Cao et al., 2004). Mammalian telomeres and subtelomeric regions are enriched in epigenetic marks that are characteristic of heterochromatin. In addition, histone deacetylase enzymes, such as Sirt6, regulate the telomeric chromatin conformation in order to allow the interaction of WRN protein with these chromosomal regions (Michishita et al., 2008).

There are evidence that point to a role of redox environment in a short term regulation of the activity of this important enzyme. Minamino et al., 2001, using vascular smooth muscle cells, reported that hypoxia up-regulates telomerase activity. Hypoxia is known to lower oxidative stress and thus to increase levels of glutathione. A specific inhibitor of telomerase, 2-[3-(trifluoromethyl) phenyl]isothiazolin-3-one, reacts with a key cysteine residue, which is essential for telomerase activity and must be kept reduced. Consequently, it has been reported that dithiothreitol reverses this inhibition (Hayakawa et al., 1999). Furthermore, antioxidants have been shown to inhibit nuclear export of telomerase reverse transcriptase and thus delay replicative senescence of endothelial cells (Haendeler et al., 2004). In conclusion, a critical cysteine residue must be kept reduced in order to maintain full telomerase activity. It is likely that the glutathione redox potential may be important in this process.

Previous findings of our group showed that telomerase is regulated by the shift in glutathione redox potential within values similar to those found *in* vivo and alterations in telomerase activity are coordinated with changes in critical cell cycle proteins, particularly Id2 and E2F4 (Borras et al., 2004).

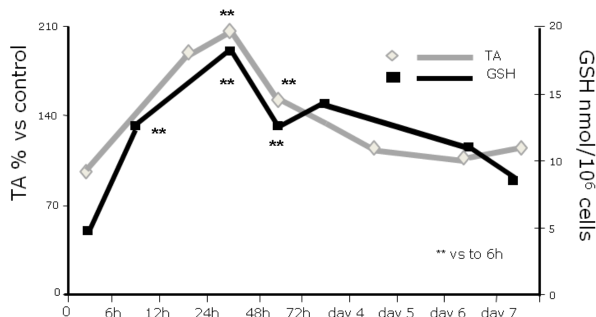

Fig. 1. Reduced glutathione regulates telomerase activity in 3T3 fibroblasts.

Thus, physiological variations in glutathione level induce changes in telomerase activities that are in concordance with changes in cell cycle regulatory proteins. A number of reports have shown similar results. Brown et al., 2007 demonstrated for the first time *in vivo* that high hepatic glutathione levels correlate with increased telomerase activity. Also, the importance of glutathione regulation in telomerase activity has been proved in endothelial progenitor cells (EPC): impairment of antioxidant defences in EPC promoted oxidant mediated apoptosis and telomerase inactivation which subsequently lead to development and/or progression of atherothrombosis (Fujii et al., 2006).

Recent data suggest that telomerase activity is regulated and ordered by telomere structure and telomerase assembly. Experimental evidence suggests that the telomere structure may change in a cell cycle-dependent manner to restrict telomerase activity to S phase (Hug & Ligner, 2006). In addition, telomere structure and specially the telomeric G-overhangs generation are strictly regulated during S phase and prolonged to other cell cycle phases depending on whether are telomeres from the lagging or the leading telomere (Dai et al., 2010). For this to happen, the precise control of the changes not only in telomerase conformation, but in chromatin structure (i.e. in its compactation level) as well, is of vital

importance. This finding that telomere length, and, therefore, telomere structure, is tightly regulated in telomerase proficient cells invokes a connection between cell cycle, telomerase and telomere structure (Blasco, 2002). In other words, the mechanism that lies beneath telomerase regulation might be related with the mechanism that control cell proliferation. This opens a highly significant area for exploratory study and the diversity of processes and control mechanisms that could be involved in this phenomenon remain to be elucidated.

3. Compartmentalization of glutathione

3.1 The physiological importance of compartmentalization of glutathione

Pioneer work from Meister, (Meister & Anderson, 1983) correlated GSH synthesis and its degradation throughout the so-called γ-glutamyl cycle, and defined it as a cytosolic processes. The importance of cellular compartmentalization of GSH is two fold, first because it plays an important role in fighting against radical oxygen species (ROS). It is well known that these molecules have a very short half life and exert their action close to the place they were produced. Thus, the presence or absence of GSH could determine the development of localized oxidative damage for the cell structure or metabolic function developed in the vicinity. Secondly, GSH compartmentalization is of vital importance because of its role as a cellular detoxifying agent; it is known that tumours that have high glutathione levels are more resistant to chemotherapy, and the importance of nuclear (Voheringer et al., 1998) and mitochondrial (Benlloch et al. 2005) compartmentalization of GSH has been pointed out.

The overview of compartmentalization of glutathione in mammal cells is a complicated matter. This is due to the presence in the literature of a number of contradictory reports. The reason for the controversy is mainly methodological. Until very recently most reports were mainly based on cell-fractionation techniques. Those techniques appear to be reliable for mitochondrial studies; however their usefulness in nuclear or even endoplasmic reticulum measurements is at least controversial.

3.1.1 Nucleus

Although the role of nuclear GSH in the synthesis of DNA (Thelander & Reichard, 1979) and in protection against oxidative damage or ionizing radiation (Biaglow et al., 1983) is well established, little is known about the concentration of GSH in the nucleus and its regulation. This is due to two main factors. The first is methodological: it is impossible to determine the nuclear concentration of GSH using standard cell fractionation and analytical approaches (for a review see Söderdahl et al., 2003). The second factor is that most, if not all, of the reports share the common view of nuclear GSH distribution in a static situation. Cells are usually studied under steady state conditions *i.e.* when they are confluent (G_0/G_1 phase of the cell cycle). The nuclear membrane dissolves during mitosis and is formed again around newly replicated DNA packed in chromosomes; this spectacular change involves a variety of regulatory mechanisms. Therefore, if the nuclear GSH distribution is studied, the cell cycle physiology should be carefully considered.

The role of GSH in cell cycle regulation has been addressed mainly from the point of view of its overall cellular content. This is surprising since it is in the nucleus where most cell cycle progression events take place. The nucleus changes dramatically during the different phases of cell cycle, and failing to consider the corresponding changes in its redox environment could confer an important disadvantage in elucidating the actual importance of glutathione in the control of cell proliferation.

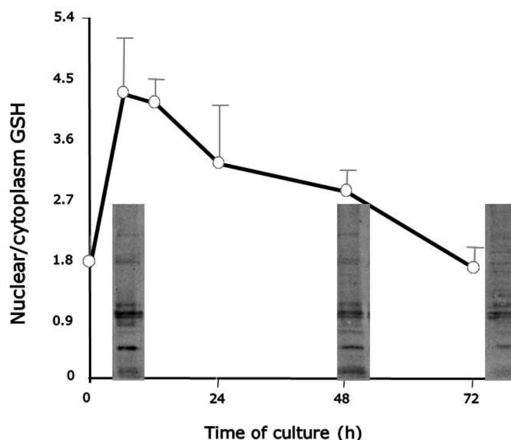

Fig. 2. Modifications of nuclear proteins along the cell cycle.

Our results, in general, are in accordance with the work of Hutter at al, (Hutter et al., 1997), who have studied the redox potential (E) in the normal and malignant cells along the cell cycle. The E of normal cells fluctuates during the cell cycle; for the proliferation to start it has to decrease at least 30mV comparing to its level in the G_0 phase (where the cells are 100% confluent). On the contrary, in cancer cells, the E remains low throughout the complete cell cycle, even at a high cell density (Hutter et al., 1997). Our evaluation of the GSH levels along the cell cycle in different models indirectly confirms these findings.
The work of Hutter et al. was further developed and completed by Hoffman et al. (Hoffman et al., 2008), who proposed recently a novel redox model of cell proliferation. They postulate the existence of a redox switch that helps regulate the proliferation within normal cells; its absence in cancer cells enables the bypass of the restriction point and leads to the loss of the control of cell cycle. The authors offer this model as a base to understand the aberrant cellular proliferation that leads to malignant transformation.
According to "redox model of cell proliferation", in normal cells exist a threshold value (θ) of $E \leq 207 \pm 11$mV which initiates the phosphorylation of different regulatory proteins associated with different phases of the cell cycle and, consequently, cell proliferation. When $E > \theta$ cell enters G1/G0 phase. However, when cancer cells are concerned, Hoffman does not take into account the fluctuations in the E level. In our hands, the increase in GSH level along the cell cycle (before the onset of the proliferation comparing to the GSH level at the final time point) is, indeed, less striking than in 3T3 fibroblasts; two-fold comparing to four-fold, respectively. The author hypothesizes, though, the existence of a reductive limit of -260mV which normal cell could not survive, but would not jeopardise cancer cell.
It was proposed previously that the proliferation occurs within the range of ROS levels, concentrations above or below this range could lead to growth arrest or cell death; hence ROS could act like a dual-edged sword (Davies, 1999).

3.2 Glutathione controls the cell cycle regulatory proteins

3.2.1 Id2 as a redox sensitive protein

The study of the expression of the Id2 along the cell cycle gave further support to this premise. The family of helix-loop-helix proteins denominated Id (inhibitor of DNA binding

or inhibitor of differentiation) was demonstrated to be of considerable importance in the regulation of cell growth, differentiation and cancer in many mammalian tissues (Norton, 2000; Yokota & Mori, 2002). Id2, in particular, was shown to disrupt antiproliferative effects of tumour suppressor proteins of the Rb family, thus allowing cell cycle progression (Lasorella et al., 1996). Indeed, the pattern of the Id2 expression detected by Western blotting, confirmed the distribution of the phases of the cell proliferation detected by flow cytometry. This observation was previously published by our group suggesting a redox regulation of this protein (Borras et al., 2004). In addition, the studies of liver regeneration, process that involves DNA synthesis and cell proliferation, gave further support to our findings.

It was demonstrated that when the increase of GSH after partial hepatectomy was prevented, the liver regeneration was delayed and the total liver amount of the DNA was lower than in the control group (Huang et al., 2001). Furthermore, an early increase in Id2 gene has been demonstrated as well as the contribution of Id2 in the control of hepatocyte priming through modulation of c-myc expression (Rodriguez et al., 2006). All these support our notion that Id2 could be an excellent candidate as a protein marker of the redox regulation of cell proliferation in our models.

3.2.2 PCNA as a possible redox sensor in the onset of DNA synthesis

PCNA, a proliferating cell nuclear antigen, is a central protein in both DNA replication and repair. It's a "sliding clamp" that localizes proteins, such as DNA polymerase, to DNA and thus enables the correct DNA replication.

Replication of mammalian genome starts at thousands of origins, called replication foci, which contain PCNA and are activated at different times during S phase. The dynamics of replication foci is still a matter of debate; there are contradictory reports on the organization of the DNA replication sites in diverse cell types attributable to the differences in the technical approach (Dimitrova & Gilbert, 2000; Kennedy et al., 2000). According to Dimitrova, SD and Berezney, R (Dimitrova & Berezney, 2002) there is no fundamental difference in the spatiotemporal organization of the DNA replication in primary, immortalized and malignant mammalian cells. On the contrary, Kennedy's group (Kennedy et al, 2000), observed different patterns of replication foci in primary versus immortalized cell lines, as well as their perinuclear localization in the contact-inhibited cells prior to cell cycle exit (Barbie et al., 2004). Another fundamental question was weather the replication foci are moving along the DNA in the process of the replication, or the DNA is spooling through fixed replication factories. It seems that the important body of evidence is accumulating supporting the fixed-replication-site model (Dimitrova & Gilbert, 2000; Leonhard et al., 2000). The replication machinary bind to DNA, but they are also tethered to an underlying framework called nuclear matrix or skeleton (Leonhardt et al., 2000). Regardless of the discrepancies in their findings, all the authors call attention to the importance of the preserving nuclear architecture in order to guarantee the correct development of the process of DNA replication (Dimitrova & Gilbert, 2000; Barbie et al., 2004; Leonhardt et al., 2000). In addition, it has been shown that chromosome territory organization depends on association with the nuclear skeleton (Leonhardt et al., 2000). More than 20 years ago, Dijkwell et al. (Dijkwell & Wenink, 1986) postulated that the maintenance of the nuclear matrix, especially nuclear lamina, by preserving disulphide bonds depended on the level of nuclear thiols. In accordance to this work, Oleinick et al. (Oleinik et al., 1987)

reported that DNA-protein cross links, present at basal level as normal associations of chromosomal loops with the nuclear matrix proteins, can be increased by ionising radiation and removal of intracellular glutathione, and decreased by hydroxyl radical scavengers. The importance of the GSH in cell proliferation could be extrapolated to the safeguarding of the nuclear architecture, providing in that way a proper milieu for the DNA replication. Our results could provide support to the hypothesis of Bellomo et al (Bellomo et al., 1997) that reduced nuclear glutathione may modulate the structural organization of chromatin. It is tempting to speculate that the high nuclear GSH level we observed before (late G1 phase) and at the onset of cell proliferation (S phase) could provide the redox environment that stimulates chromatin decompaction by reducing disulfide bonds.

3.3 Nuclear compartmentalization of glutathione as an important feature of proliferating cell: reduce to replicate

The functional compartmentalization is an obvious characteristic of eukaryotic cell. The organelles, visible by light microscopy, are surrounded by membranes, which, although permitting communication, provide unique and defined environment in each one, which guarantee its accurate function. Probably the most remarkable examples of compartmentalization are oxidative phosphorylation in mitochondria, protein folding in endoplasmic reticulum and, for the purpose of this study the most interesting of all, DNA synthesis. It is interesting to note that, when the first two organelles are concerned, the dependence of their function on the correct GSH level has been thoroughly studied. The high intramitochondrial concentration of GSH is maintained by an active multicomponent transport system "pumping" glutathione from the cytosol into the matrix (Matensson et al., 1990). On the contrary, for the correct folding of proteins into a native structure by disulfide bond formation, the GSH level and the ratio GSH/GSSG in the endoplasmic reticulum is maintained at extremely low level by the limited permeability of the vesicle membrane to GSH (Hwang et al., 1992). However, in the case of nuclear compartmentalization of glutathione the reports were scarce and contradictory over the years. This could be attributed to two main factors: methodological difficulties in measuring nuclear glutathione content and the focus of the research generally limited to confluent cells.

3.4 Modifications of nuclear proteins along the cell cycle

Various studies have demonstrated that the nucleus is more reduced than the cytosol (15mM GSH vs. 11 mM, respectively) (Bellomo et al., 1997; Schafer & Buettner, 2001; Soboll et al., 1995). An important number of nuclear proteins, including transcription factors, require a reduced environment to bind to DNA. More than 62 proteins are involved directly in transcription, nucleotide metabolism, (de)phosphorylation, or (de)ubiquitinylation, which are all essential processes for cell cycle progression (Connour et al., 2004). For instance, it appears that, at the onset of cell proliferation in the early G1 phase, an increase of ROS in the cytoplasm is necessary for the initiation of the phosphorylation cascade mediated by epidermal growth factor (EGF) that, subsequently, activates DNA replication and the cell division (Carpentes & Cohen, 1990). According to Jang and Surh (Jang & Surh, 2003) nuclear GSH may act as a transcriptional regulator of NF-κB, AP-1, and p53 by altering their nuclear redox state. The transcription factor NF-κB is an example of distinct redox-sensitive activation and DNA binding (Hansen et al., 2006); it is activated by various physiological stimuli known to produce ROS; on the contrary, to permit DNA binding, similar to Fos, Jun,

and Nrf2, cysteine residue within DNA binding domain must be reduced. Both processes are guaranteed by the adequate redox state of the cytosolic and nuclear environment, respectively.
Interestingly, the nuclear proteins underwent stronger glutathionylation before and at the onset of cell proliferation than at quiescence. It is not surprising if we bear in mind that high level of GSH in the nucleus could provide protection to the proteins against the oxidative threat coming from the cytoplasm at the early phase of cell proliferation, and that glutathiolation, as it is a reversible modification, could be just the way. On the other hand, based on the simplicity of the redox transition from thiol to disulfide and on the fact that the reversibility was energetically favourable, Cotgrave IA and Gerdes RG (Cortgreave & Gerdes, 1998) more than 10 years ago have proposed glutathionylation as a posttranscriptional modification with the regulative finality. They state that it offers "a strong possibility for transducing "oxidative information" from intracellular oxidants via the GSH redox buffer to individual proteins containing "regulatory thiols". Also, recently, this posttranslational modification was proposed as a likely molecular mechanism for redox dependent signalling mediated by GSH (Fratelli et al., 2005). Thus, high level of GSH in the nucleus, observed before and at the onset of cell proliferation, could provide the "GSH redox buffer" necessary for the progressing of oxidant stimulated mitogenesis. It is encouraging to see that the findings of the present study have provided some support to the assumption that a dynamic intracellular redox environment directs the cell proliferation through redox sensitive cell cycle proteins.

4. The depletion of nuclear glutathione hampers the cell cycle progression

With the intention of providing further evidence of the importance of nuclear GSH in the initiation of cell proliferation, we have found ourselves in front of a challenge of depleting nuclear glutathione. A number of reports have focused on the consequences of the depletion of cellular glutathione levels on changes in cellular proliferation (Thomas et al., 1995; Hansen et al., 2006). However, all those reports were performed measuring cellular or total glutathione levels, but there is no information relating cellular proliferation with nuclear glutathione levels. A number of studies have indicated the existence of a nuclear GSH pool that resists depletion after exposure of cells to BSO (Thomas et al., 1995). BSO treatment resulted in the concentration dependent depletion of cytoplasmic GSH, while the depletion of mitochondrial and nuclear pool of GSH required concentrations higher than 100 µM, which induced DNA damage (Green et al., 2006). Spyrou and Holmgren (Spyrou & Holmgren, 1996) showed that inhibition of glutathione synthesis by 0.1 mM BSO was able to decrease GSH synthesis after treatment for 12 hours, but GSH-depleted cells grew as well as control 3T6 cells with no decrease in DNA synthesis. Thus, incubation of cells with low concentration of BSO, although decreases glutathione levels, does not change cell proliferation. On the other hand, Thomas *et al.* (Thomas et al., 1995) showed that non toxic concentrations of N-ethyl maleimide or DEM decreased the GSH level in the nucleus and cytoplasm to a similar extent, whereas the nuclear pool of GSH was much more resistant to BSO depletion.
Based on this findings, we have designed a model to study the effects on the cell proliferation parameters caused by GSH depletion both in the nucleus and the cytoplasm, using 100µM DEM, comparing to the administration of 10 µM BSO when nuclear GSH level is preserved.

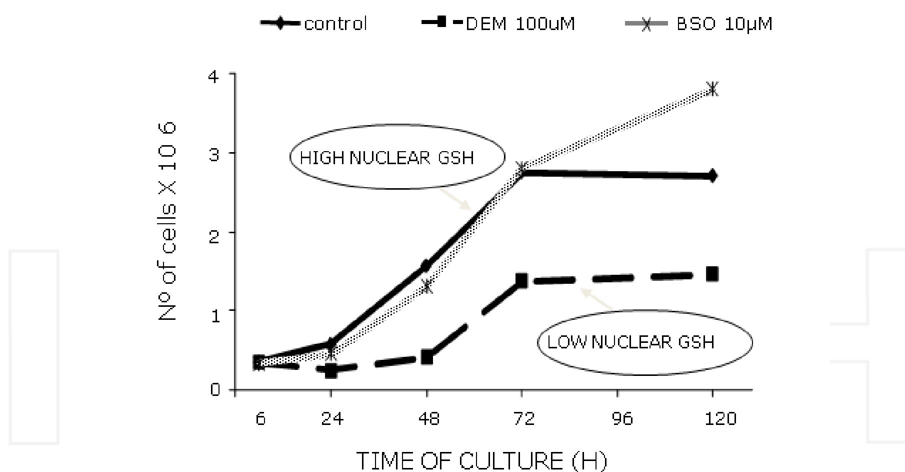

Fig. 3. Changes in cell proliferation caused by GSH depletion.

As reported previously by various authors (Britten et al., 1991; Green et al., 2006; Thomas et al., 1995) nuclear GSH pool was preserved. By contrast, depletion of glutathione levels by DEM induces a marked decrease in nuclear glutathione levels.

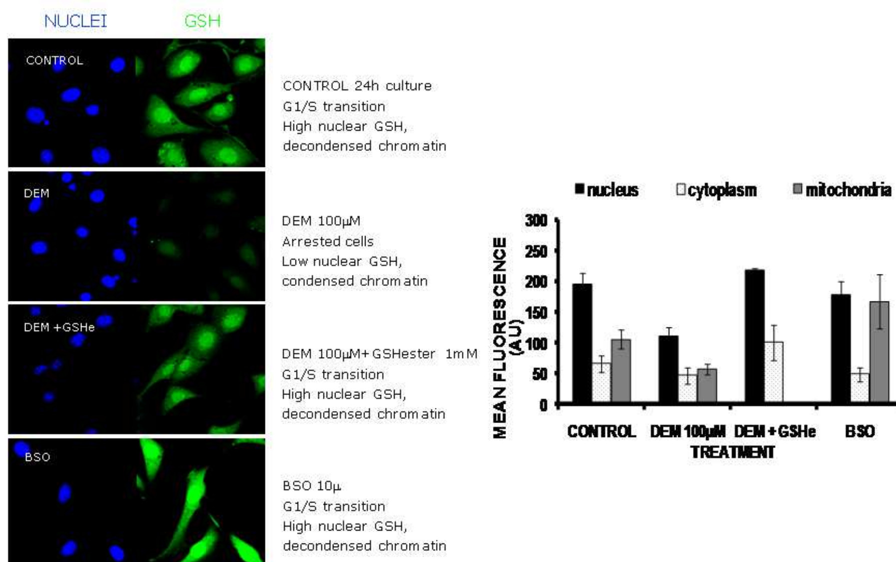

Fig. 4. The depletion of GSH in the nucleus.

The compartmentalization of glutathione depletion could explain the observed differences in the inhibition of cell proliferation. Indeed, our results show that inhibition of glutathione

levels by DEM strongly impairs cell proliferation. This difference could be due to the fact that DEM decreases both nuclear and cytosolic glutathione levels in opposition to BSO, which only decreases cytosolic glutathione. It is worth mentioning that the impairment of cell proliferation could not be attributed to the alkylating properties of DEM, since the simultaneous administration of GSHe completely prevented it, nor to the toxicity of the treatment because the cell death was not significantly augmented.
In addition, we have observed the delay in the cell cycle progression caused by DEM, when both nuclear and cytoplasmic GSH was depleted, which is absent in the treatment with BSO when nuclear GSH pool was preserved. Interestingly, Esposito *et al*. (Esposito et al., 2002) showed that direct administration of DEM on the nuclear extracts of COS7 cells induces cell cycle arrest. So, it is daring to speculate that, despite the depletion of cytoplasmic GSH with DEM could not be overlooked, the effect on the cell cycle progression could be attributed to the depletion of the nuclear GSH. Moreover, as Esposito shows, the depletion of nuclear GSH strongly induces a p53-independent accumulation of p21, which causes a cell cycle arrest. In our study, the expression of cell cycle regulatory protein, suggested previously to be under redox control, Id2, was decreased when the level of nuclear GSH was depleted.

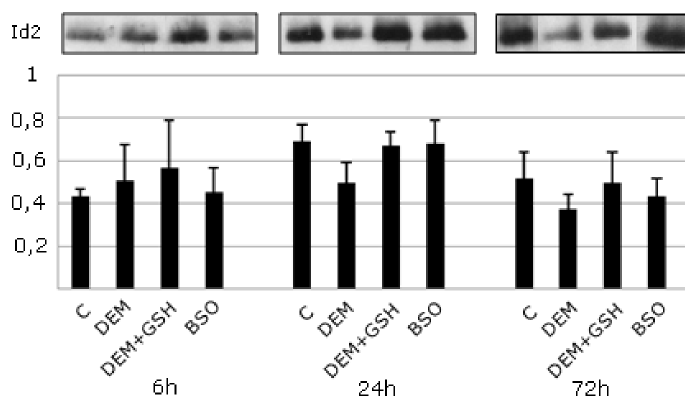

Fig. 5. Nuclear GSH depletion affects Id2 expression.

It has been known that cell proliferation is regulated by a variety of mechanisms working to allow the activation and repression of growth stimulatory genes, one of them being the transcription factors. Previous *in vitro* reports show that the activity of transcription factors is related to its redox environment. In addition, change in the redox potential could induce variations in the activity of those transcription factors. Alterations as small as $\pm$ 15 mV in the redox potential can result in transcription factor translocation and activation or deactivation, depending on the direction of the redox shift (Hutter et al., 1997; Sen & Packer, 1996; Sun & Oberley, 1996). Recently Reddy *et al.* (Reddy et al., 2008) have shown that Nrf2 deficiency leads to oxidative stress and DNA lesions, accompanied by impairment of cell-cycle progression, mainly G(2)/M-phase arrest. Both N-acetylcysteine and glutathione (GSH) supplementation ablated the DNA lesions and DNA damage-response pathways in Nrf2 (-/-) cells; however only GSH could rescue the impaired co-localization of mitosis-promoting factors and the growth arrest. Our results demonstrate for the first time that it is

nuclear GSH levels, and not total cellular glutathione levels, that specifically correlate with cellular proliferation. Glutathione is considered essential for survival in mammary cells and other eukaryotic cells, but not prokaryotic cells. However, although a number of important functions have been attributed to GSH, its outstanding role in nucleated, but not in prokaryotic cells, remains unknown. Our results underscore the important role of nuclear glutathione in cell physiology and suggest that manipulation of nuclear GSH levels could be of paramount importance during development and cancer.

5. The occurrence of the glutathione in the nucleus; active transport, de novo synthesis, diffusion or something else

How GSH enters the nucleus and how it is regulated during the different phases of the cell cycle is still a matter of debate. The regulation of such interactions is also unclear. According to Smith and colleagues (Smith et al., 1996), the possible biochemical mechanisms responsible for the turnover of nuclear GSH are the following: 1.GSH may be taken up from the cytoplasm into the nuclei either passively or through energy-dependent processes 2.GSH may be synthesized *de novo* in the nucleus by the enzymes glutamate cysteine ligase and GSH synthetase 3.GSH may function to transport γ-glu-cys-cys.

5.1 ATP dependent sequestration of the GSH to the nucleus

The role of ATP-dependent mechanisms in maintaining the nuclear/cytoplasmic GSH concentration in hepatocytes was demonstrated by Bellomo and co-workers (Bellomo et al., 1997). After 20 min of incubation with the uncoupler protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) the nuclear/cytoplasmic GSH gradient disappeared, but the total GSH content remain unchanged. This study was questioned by Briviba et al (Briviba et al., 1993) because of the use of monochlorobimane (BmCl). Despite its high specificity for glutathione this fluorochrome was found to be of no value in the study of cellular GSH distribution; once GSH-BmCl conjugate is formed it demonstrates an increased tendency of nuclear compartmentalization. Indeed, in our study using CMFDA, we have not found an ATP-dependent mechanism of nuclear GSH compartmentalization in 3T3 fibroblasts. Ho and Guenthner, 1997 using nuclear fractions concluded that GSH is taken up by the nucleus by passive diffusion and no evidence for an ATP-dependent mechanism for GSH concentration was observed.

5.2 Nuclear synthesis of glutathione

Glutamate cysteine ligase (GCL) and GSH synthetase activities have been reported in nuclei, and a portion of 4-8% of cell GSH synthetic activity is considered capable of maintaining nuclear GSH levels (Ho and Guenthner, 1997). However, we could not find GCL expression in nuclei of 3T3 fibroblasts. In addition as previously reported BSO, a specific inhibitor of GCL was unable of decreasing nuclear glutathione levels. Thus, at least under our experimental conditions, the possible “de novo” synthesis of nuclear glutathione seems improbable in 3T3 fibroblasts.

5.3 GSH enters nucleus via nuclear pores

The nuclear pore complex is the biochemical machinery that controls the molecular traffic across the nuclear envelope (Feldherr & Akin, 1990; Nigg, 1997). Ions and small hydrophilic

molecules, like glutathione, are considered to move by free and fast diffusion across the nuclear pore (Ribbeck & Gorlich, 2001); nevertheless ion gradients and transnuclear ATP-dependent membrane potential have also been reported (Nigg, 1997). In a series of creative experiments published in early 1990ies, Feldherr CM and Akin D (Feldherr & Akin, 1990; Feldherr & Akin, 1993), shown that permeability of nuclear envelope and nuclear transport were higher in proliferating than in quiescent cells. Reported seven fold reduce in the nuclear transport capacity was induced by the alterations in the characteristics of the pores and not by the changes within the cytoplasm, specifically, the decrease in ATP concentration. One pore forming protein that has been brought into the connection to nuclear glutathione content is Bcl-2. Voehringer and colleagues (Voehringer et al., 1998) showed that over-expression of Bcl-2 recruits GSH to the nucleus. The presence of this protein at the nuclear envelope was demonstrated (Krajewski et al., 1993) and the association with the nuclear pore complexes was suggested. Moreover, Zimmermann et al. (Zimmermann et al., 2007) demonstrated that GSH binds to Bcl-2 in mitochondria, providing a molecular basis for its antioxidant function.
A clear picture emerges showing that the presence of a reduced nuclear environment, probably provided by glutathione, glutaredoxin and thioredoxin mainly, is of paramount importance in the physiology of cell cycle, underscoring the role of oxidative stress in cell proliferation.

6. References

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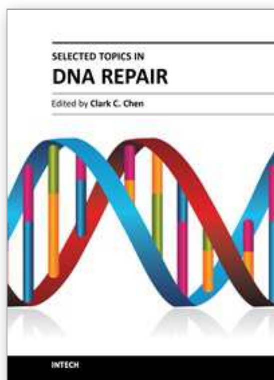

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This book is intended for students and scientists working in the field of DNA repair, focusing on a number of topics ranging from DNA damaging agents and mechanistic insights to methods in DNA repair and insights into therapeutic strategies. These topics demonstrate how scientific ideas are developed, tested, dialogued, and matured as it is meant to discuss key concepts in DNA repair. The book should serve as a supplementary text in courses and seminars as well as a general reference for biologists with an interest in DNA repair.

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