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Transcripts of Damaged Genes in the Brain During Cerebral Oxidative Stress

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Abstract

Recent studies using ischemia/reperfusion models of brain injury suggest that there is a period of time during which the formation of oxidative DNA lesions (ODLs) exceeds removal. This interval is a window of opportunity in which to study the effect of gene damage on gene expression in the brain, because the presence of excessive ODLs mimics a deficiency in gene repair, which has been shown to be associated with neurological disorders. Evidence from studies using similar models indicates that expression of faulty transcripts from ODL-infested genes and non-sense mutation in repaired genes occur before the process of cell death. Preventing the formation of ODLs and enhancing ODL repair are shown to increase the expression of intact transcripts and attenuate cell death. Understanding this mechanism could lead to the development of therapeutic techniques (physiologic, pharmacological, and/or genomic) that can enhance recovery.

Keywords

head injury; immediate early genes; mutagenesis; neurodegeneration; nitric oxide; oxidative stress; repair; signal transduction; stroke

The emerging field of functional genomics has identified various types of germline mutations that may be associated with neurologic disorders. For example, mutations occur in the α -synuclein gene in familial forms of Parkinson's disease (Solano et al., 2000) and the superoxide dismutase gene in familial amyotrophic lateral sclerosis (D. Liu et al., 1999; Cha et al., 2000; Guo et al., 2000). Gene mutations that have been found include base substitutions, deletions, and frameshifts that cause the null expression of proteins (nonsense mutation). Although familial forms of neurologic disorders affect thousands of people, germline mutations account for a small percentage of those afflicted with the disease, most of whom have sporadic forms. Studies in other established disciplines (e.g., carcinogenesis and functional genetics) show that genetic mutation can be induced in somatic cells (somatic cell mutagenesis), accounting for those with sporadic forms of the disease. With the exception of X chromosome-linked diseases, two mutational steps are typically required to inactivate a genetic locus, because there are two copies of each chromosome in each somatic cell. Recent studies show that brain injury of the ischemia/reperfusion type, as well as impact head injury, induces gene damage in the brain (Liu et al., 1996; Clark et al., 2001). Although gene damage is repaired by a relatively error-

free mechanism, brain injury induces an increase in the baseline genetic damage, which may be beyond the capacity for repair (Cui et al., 1999b; Moore et al., 2002). Transient deficiency of gene repair after brain injury may cause adverse effects similar to those in humans (de Boer et al., 2002). These findings suggest that experimental models of brain injury can be used to study initiating factors that cause genetic alternation after cerebral oxidative stress.

Brain injury of the ischemia/reperfusion type induces cerebral oxidative stress, which occurs after stroke and cardiac arrest and is the leading cause of neurologic disability in the United States. Clinical observation and research using experimental brain injury models have implicated several initiators in the cascade of events that lead to neuronal degeneration; these include decreased levels of intracellular ATP (energy depletion) and pH and increased levels of extracellular glutamate, intracellular calcium, proteases, and reactive oxygen species (ROS; Zhang et al., 1995; Kumura et al., 1996; Brorson et al., 1999; Lipton 1999; Lewen et al., 2000). The increases in intracellular calcium levels, ROS, and extracellular excitatory amino acids occur early and simultaneously (Katayama et al., 1991; Kunkler et al., 1998). Gene damage is increased immediately after ischemia/reperfusion, whereas nuclear gene mutation and neuronal death are detected within hours after the onset of cerebral ischemia (Liu et al., 1996). Mechanisms of cell death in the brain have been studied extensively, however, little research has been directed toward functional genomics in damaged genes of the surviving brain cell population. Somatic cell mutagenesis (probably resulting from repair deficits or errors during gene repair) in combination with germline mutations may play a significant role in preventing cellular restoration and functional recovery among individuals who incur neurologic insults in which elevated cerebral oxidative stress is also known to occur (Reardon et al., 1997). Genetic analysis may eventually help in explaining the variability in functional outcomes (Yoshimura et al., 1998; Nakayama et al., 1999).

With the exception of ionizing radiation and pathological conditions under which the blood—brain barrier is disrupted, many environmental agents that induce cellular oxidative stress in tissue do not interact with brain cells. Cerebral oxidative stress, however, can be induced in several animal models that simulate stroke and cardiac arrest and has been reviewed in detail elsewhere (Lipton, 1999). We have focused on nuclear DNA as a critical target for oxidative damage because the essential genes are present in only one or two copies in each mammalian cell. Here we review current knowledge on gene damage in the brain after cerebral oxidative stress and the effect of gene transcripts from genes that are infested with oxidative damage. We then review the effect of gene repair errors, a phenomenon that occurs after brain injury.

NITRIC OXIDE AND OXIDATIVE STRESS

Brain injury causes an increase in extracellular glutamate as a result of the activation of a certain subclass of glutamate receptors (N-methyl-D-aspartate or alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; for review see Yamada, 2000; Kontos, 2001). Glutamate receptor activation causes a massive influx of calcium, which, in turn, activates neuronal nitric oxide synthase (nNOS) and inducible NOS (iNOS; Dawson et al., 1991). There is an increase in the nitric oxide (NO) level in brain tissue following cerebral ischemia/reperfusion, traumatic head injury, and spinal cord injury (Zhang et al., 1995; Jones et al., 1995; Kumura et al., 1996; Cherian et al., 2000). There is also evidence indicating that the level of superoxide ion is elevated after brain injury (Chan et al., 1987; Liu et al., 1989).

The nitrogen radical that is produced by enhanced nNOS activity has dual fates. First, it can saturate superoxide dismutase and inhibit its reducing effect on superoxide ion (Estevez et al., 1998); second, it can combine with superoxide ion ($O_2^{\bullet-}$) to form peroxynitrite (Beckman et al., 1990). 3-Nitrotyrosine, a marker of peroxynitrite, is elevated after cerebral oxidative stress in various disease states (Smith et al., 1997; Eliasson et al., 1999; Scott et al., 1999). Recent

studies show that NO radical and superoxide anion, independently or in combination, may contribute to nuclear gene damage after cerebral ischemia (Cui et al., 2000; Huang et al., 2000). Less is understood concerning mitochondrial gene damage, although there is excellent evidence showing its presence after brain injury (Cui et al., 1999b; Englander et al., 1999; Chen et al., 2001). Mice with a knockout of nNOS and ordinary animals treated with 7-nitroindazole (7NI; an inhibitor of nNOS) are protected against neuronal death after experimental brain injury of the ischemia/reperfusion type (Kamii et al., 1994; Huang et al., 1994; Yoshida et al., 1994; O'Neill et al., 1996; Panahian et al., 1996; Iadecola et al., 1997). Reducing the levels of NO and/or superoxide ion attenuates neurotoxicity after brain injury (for review see Lewen et al., 2000). Although the neuroprotective effect in the nNOS knockout animals may be confounded by an alteration of endothelial NOS activity (Iadecola et al., 1997; Santizo et al., 2000), it is clear that the increased activity of nNOS induced by brain injury is neurotoxic via the formation of 3-nitrotyrosine and/or gene damage (Eliasson et al., 1999; Cui et al., 1999b, 2000; Huang et al., 2000).

GENETIC DAMAGE

The brain has the highest metabolic rate of all organs and depends predominantly on oxidative metabolism for its energy. Metabolism of oxygen generates ROS, which can interact with all cellular components, including protein, lipids, and nucleic acids. Thus, ROS cause damage to membranes, RNA, DNA, and inactivate enzymes and transcription activators (Salminen et al., 1995; Liu et al., 1996; Lipton, 1999; Lewen et al., 2000). ROS generated by cerebral oxidative stress interact with nucleic acids and cause the formation of oxidative DNA and RNA lesions (ODLs and ORLs, respectively; Floyd and Carney, 1992; Cui et al., 1999b; Huang et al., 2000).

There are more than 70 different forms of ODLs and ORLs, eight of which have been demonstrated in the brain using several models of brain injury (for review see Liu et al., 2001). Five are base modifications, and three are single-stranded breaks (SSBs), and they occur in neurons, as well as in astrocytes, at the beginning of reperfusion (Cui et al., 1999b; Huang et al., 2000). Thus, the time after injury when ODLs and ORLs are elevated coincides with the time when excitatory amino acids are released and calcium influx occurs (Shimizu-Sasamata, 1998; Kunkler et al., 1998). Although SSBs will induce effects similar to those of base modifications, here we considered the effect of base modifications, because SSBs that are detected in the brain after injury can occur from DNA synthesis during repair process and cell replication (neurogenesis), which are considered normal cell functions.

ODLs cause a change in its coding properties during DNA and RNA synthesis (replication and transcription, respectively) and eventually will affect protein synthesis (translation; de Boer et al., 2002; for review see Wood et al., 2001; Friedberg et al., 2002). Some ODLs and ORLs terminate chain elongation during transcription and translation (Hanawalt, 1994). Finally, neuroinflammatory processes that induce iNOS in response to the initial injury may further delay recovery and cause brain cell death (Floyd, 1999; Kim et al., 2001). The immediate and long-term effects of gene damage in the brain after injury are discussed below.

GENE REPAIR

Under physiologic conditions, DNA repair processes remove ODLs in mitochondrial and nuclear genes (Liu et al., 1996; Love et al., 1998; Chen et al., 2000; Lin et al., 2000; De Souza-Pinto et al., 2001). Gene repair processes normally make few errors in the repair of ODLs and are very important for cell function. Because ODLs occur relatively soon after the injury and can be reduced by repair processes, gene damage may easily escape detection. New assays have been developed to detect ODLs in nuclear genes of the brain (for review see Liu et al., 2001). ODLs are present in all three genes (actin, c-fos, and DNA polymerase- β genes) that

have been assayed using an experimental stroke model in the rat and a cardiac arrest model in the mouse (Liu et al., 1996; Moore et al., 2002). All of these genes are actively transcribed in the brain; therefore, it is most likely that other active genes are targets of ROS. Although the number of ODLs in each of the three genes could be different initially (Fig. 1), the rate of repair increases linearly up to 45 min and remains steady thereafter (Cui et al., 1999b; Moore et al., 2002). The half-life of ODLs in these nuclear genes is 30 min. Furthermore, approximately 15–20% of the initial ODLs had not been repaired at the end of 120 min of reperfusion. This observation confirms observations made using other methods (Dizdaroglu, 1992; Floyd and Carney, 1992).

Enzymes that repair genes have been reviewed elsewhere (for review see Wood et al., 2001; Friedberg et al., 2002). Two repair enzymes that remove some forms of ODLs are 8-oxo-2'-deoxyguanosine glycosylase and endonuclease APE/Ref-1, and their activities are elevated after brain injury (Fujimura et al., 1999; Lin et al., 2000). DNA-dependent kinase is also reported to be elevated after brain injury (Shackelford et al., 1999). However, the burden of genetic damage exceeds the repair capacity of the brain during the first hours of reperfusion (Fig. 1). Investigations by others have shown that different regions of the brain may be preferentially targeted for oxidative gene damage and have different rates of repair (Cardozo-Pelaez et al., 2000). Indeed, very few neuroprotective transcripts are significantly expressed during the first hour of reperfusion (An et al., 1993; Schmidt-Kastner et al., 1998; for review see Sharp et al., 1999). Furthermore, tissue repair via neurogenesis in the brain occurs at extended reperfusion times and is reported to occur in specific cell populations (J. Liu et al., 1998; Takagi et al., 1999; Gu et al., 2000; Jiang et al., 2001; Jin et al., 2001; Arvidsson et al., 2001; Yoshimura et al., 2001). To maintain gene expression and replenish the protein that has been inactivated by proteases after injury, the brain would have to contain an extremely accurate mechanism by which to repair existing molecules, such that oxidative damage occurring in an oxygen-utilizing organ would be kept to a minimum. Therefore, molecular repair of genes is essential to cell restoration in the brain. Very little is understood about how ODLs are removed and repaired in the brain.

EFFECTS OF GENE DAMAGE

Relatively little is known about the effect of ODLs during the primary insult, when the level of ODLs is elevated. Expression of immediate early genes (IEGs) is stimulated during initial hours as a part of the endogenous responses to injury or stimuli. The burden of genetic damage may delay and/or inhibit this neuroprotective response (Fig. 2). In the following sections, we explore immediate and long-term effects of ODLs: 1) energy depletion, 2) compromised expression of transcription activators during the early reperfusion period, and 3) mutagenesis in surviving cells.

Gene Damage Activates Proteins that Cause Energy Depletion

Cellular injury in the ischemic core appears to be directly related to energy depletion (Eliasson et al., 1997). DNA SSBs activate poly(ADP-ribose) polymerase (PARP) after focal cerebral ischemia/reperfusion (FCIR). This activation occurs as early as 5 min after reperfusion, following 2 hr of ischemia (Endres et al., 1997). Treatment with 3-aminobenzamide (an inhibitor of PARP) or 7NI reduces PARP activation (Tokime et al., 1998). Overall, NO-mediated DNA damage can activate PARP, leading to energy depletion and necrosis (Zhang et al., 1994; Royland et al., 1999; Mandir et al., 2000; Ying et al., 2001). Thus, pharmacologic inhibition of peroxynitrite and reduction of ODL production may prevent energy depletion.

On the other hand, direct inhibition of PARP using 3-aminobenzamide in a transient global ischemia model of stroke causes an accumulation of SSBs in neural tissue. This accumulation is thought to occur by preventing DNA ligation during the last step of gene repair, resulting in

an increase in apoptotic cell death (Nagayama et al., 2000). These observations are consistent with the requirements for energy and the expression of certain genes in apoptotic process (for review see Floyd, 1999).

Transcription of Genes With ODLs Produces Faulty Transcripts

After brain injury, activation of membrane receptors can be mediated via different G proteins, leading to the activation of phospholipases, phosphoinositide hydrolysis, and formation of inositol triphosphate and diacylglycerol. This event leads to Ca^{2+} influx and activation of protein kinase C and growth factors (Avossa and Pfeiffer, 1993; Cui et al., 1999a). Other membrane receptors may change adenylate cyclase and cAMP production. It is likely that membrane receptor activation leads to activation of MAP kinase, which regulates the expression of several genes. Spreading depression (or depolarization) in the tissue surrounding the core, in combination with glutamate receptor-mediated neurotransmitter release, intracellular calcium accumulation, and/or NOS activation, induces gene expression in the penumbra (Malinski et al., 1993; Kano et al., 1998; Shimizu-Sasamata et al., 1998; Dietrich et al., 2000; Xu et al., 2001; Katano et al., 2001). Gene transcription is suggested to be a critical step in maintaining neuronal viability (Hata et al., 2000b). Examples include increased expression of IEGs (c-fos, heat shock proteins, c-jun, cAMP-responsive element binding), neurotrophin genes (nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3), and cell cycle regulator genes (bcl-2, p53; Sagar et al., 1988; Sharp et al., 1991; An et al., 1993; Salminen et al., 1995; Akins et al., 1996; Fujimura et al., 1999; P.K. Liu et al., 1999). The number of genes whose transcripts can be detected is growing as a result of microarray technology. However, the exact function of these transcripts is not understood completely.

Recent studies show that gene transcription occurs when the gene contains massive ODLs (Cui et al., 1999b; for review see Y. Liu et al., 1998). Most DNA lesions induced by environmental mutagens block gene transcription (for review see Hanawalt, 1994; de Boer et al., 2002), except ODLs that are induced by ionizing radiation and ROS (Viswanathan et al., 1999; Tornaletti et al., 2001). To study gene expression after brain injury, many investigators, including ourselves, have selected the *c-fos* gene, because its gene product, Fos, is a major component of activator protein-1 (AP-1). AP-1 is a transcription factor, and neurotoxicity of AP-1 has been implied by several studies. Fos/AP-1 is expressed at a minimal level under normal conditions but is activated immediately after stroke (An et al., 1993). Recent studies show that mutant *c-fos* mRNA is transcribed when its nuclear gene contains ODLs (Cui and Liu, 2001). The expression of mutant *c-fos* mRNA occurs before the activation of the apoptosis pathway (Liu, 2002).

Specific inhibitors of nNOS are neuroprotective when applied early in reperfusion. Inhibition of nNOS during the ischemia phase in stroke or cardiac arrest models reduces the burden of ODLs detected during the early reperfusion interval (Cui et al., 1999b; Huang et al., 2000). It enhances the early expression of intact *c-fos* mRNA in the ischemic cortex and dentate gyrus of the brain (Cui and Liu 2001; Liu et al., 2002). The elevated expression of intact *c-fos* mRNA during the early reperfusion interval in the presence of an nNOS inhibitor may be related to reduced production of NO (Eliasson et al., 1999) and, secondarily, an enhancement of processes that repair ODLs (Fig. 3). Thus, limiting the production of ROS and enhancing repair processes, together and/or independently, break the cycle of events that leads to a build-up of ODLs and energy depletion, thereby allowing for the earlier expression of intact neuroprotective and neurogenic proteins (Bansal and Pfeiffer, 1997). Several questions remained to be answered. What is the effect of faulty gene transcription in acute forms of injury? Can it affect translation during the first hour of reperfusion (An et al., 1993; Schmidt-Kastner et al., 1998)? Can its translation upset late gene activation and lead to a reversed ratio of anti- to proapoptotic protein?

Gene Damage Enhances Somatic Cell Mutagenesis

The long-term effect of excessive ODLs/ORLs that are not repaired or incorrectly repaired is positively related to mutation, delayed neurogenesis, and cell death (Liu et al., 1996; Fujimura et al., 1999; Moore et al., 2002; for review see Hanawalt, 1994; Friedberg et al., 2002). An example is frameshift mutations in the *lacI* gene of the Big Blue mouse brain after forebrain ischemia/reperfusion injury (Liu et al., 1996). These mutations result in the null expression (LacI peptides) of this reporter gene. Studies show that mutation in a CCC triplet is specifically induced by oxidative stress (Newcomb and Loeb, 1998). This type of mutation in the CCC triplet has not been detected under physiologic conditions. We have observed an increased burden of mutations in nuclear genes of the brain within 8 hr after cerebral ischemia (Liu et al., 1996). The mutation that is detected includes deletion and frameshift mutations of the *lacI* gene in a transgenic mouse strain (the Big Blue strain). In this study, 83% of the base substitution mutations occurred in GC pairs, and a single type of base mutation was found in all C nucleotides of a triplet CCC (8.3% of all induced mutations). Therefore, cerebral oxidative stress after brain injury mimics what occur in other human tissue after exposure to gene-damaging agents. Mutations in naturally occurring genes of brain cells have not been reported after brain injury but are believed to occur (Nakayama et al., 1999; Liu, 2002). Because acute energy depletion causes null expression of gene products (Hata et al., 2000a), can somatic cell mutation after injury of the brain that causes null expression of certain genes mimic a situation of chronic energy depletion?

CONCLUSIONS

Oxidative stress caused by ischemia/reperfusion affects all cellular components, but none with a more profound impact than damage to DNA in the neonates especially. This is especially true when a germline mutation is already present. Cellular and regional sensitivity to this oxidative damage may have clinical implications in terms of specific functional deficits after an ischemia/reperfusion insult. NO and other ROS are the key initiators of ODLs. Future research should focus on pharmacologic, physiologic, and genomic interventions in NO and ROS production and distribution, such that oxidative gene damage, which slows or prevents the endogenous neuroprotective response, is minimized. The expression of Fos/AP-1 immediately after FCIR is cited here as an example. The ways in which cells escape (or are unable to escape) the effect of oxidative damage may depend on additional IEGs and subsequent gene expression (Sharp et al., 1999; Tamatani et al., 2001). Understanding the types and consequences of unrepaired lesions that result in mutation is critical to understanding ways to prevent or repair somatic cell mutation. If one could optimize the timing and extent of the endogenous protective response mediated through IEGs and late effector genes, one could theoretically improve functional outcomes among patients sustaining ischemia/reperfusion insults as well as those patients with chronic, progressive, neurologic disease (Parkinson's disease, amyotrophic lateral sclerosis).

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Fig. 1.

Presence of ODLs in three nuclear genes of the brain after FCIR. [Combined data from Cui et al., 1999b; Moore et al., 2002.]

Fig. 2.

Hypothesis of ODLs causing faulty gene transcription and the failure to activate late effector genes. One particular pathway presented here is the activation of neurotrophin by the Fos/AP-1 complex, products of the *c-jun* and *c-fos* supergene families. Damage to the *c-fos* gene can be reduced via gene repair or inhibitors/scavengers that reduce reactive oxygen species (Liu et al., 2001). NTGs, neurotrophin genes; FGF, fibroblast growth factor; PARP, poly(ADP-ribose) polymerase.

Fig. 3. Repair activities are further enhanced in the presence of nNOS inhibition. Forebrain ischemia/reperfusion-induced mouse 8-hydroxy-2'-deoxyguanosine (oh8dG) glycosylase activities that remove oh8dG in brain extracts in four groups of animals were measured by the excision of oh8dG (X in 5'-³²P-CATCATGGTCXTGGTTTGGGCA-3'; see Lin et al., 2000).