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**High Concentrations of Excised Oxidative DNA Lesions in Human Cerebrospinal Fluid, Rafał Rozalski,<sup>1</sup> Piotr Winkler,<sup>2</sup> Daniel Gackowski,<sup>1</sup> Tomasz Paciorek,<sup>1</sup> Heliodor Kasprzak,<sup>2</sup> and Ryszard Olinski<sup>1\*</sup>** (<sup>1</sup> Department of Clinical Biochemistry, The Ludwik Rydygier Medical University in Bydgoszcz, Karłowicza 24, 85-092 Bydgoszcz, Poland; <sup>2</sup> Clinic of Neurosurgery, The Ludwik Rydygier Medical University in Bydgoszcz, Skłodowskiej-Curie 9, 85-092 Bydgoszcz, Poland; \* author for correspondence: fax 48-52-585-3771, e-mail ryszardo@aci.amb.bydgoszcz.pl)

The high rate of oxygen consumption per unit mass of tissue renders the brain especially vulnerable to the deleterious effects of oxidative stress, which can arise from the overproduction of reactive oxygen species or from a deficiency of the antioxidant defense systems. Reactive oxygen species have the potential to modify all four DNA bases. Production of 8-hydroxyguanine (8-OH-Gua) bases one of the most critical lesions of this type (1–3).

Products of DNA damage repair are excreted into the urine or other extracellular fluids without further metabolism (4–6). The rates of excretion of 8-OH-Gua and 8-hydroxy-2'-deoxyguanosine (8-OH-dGuo; modified base and nucleoside, respectively) in urine may be useful indicators of oxidative DNA damage and reflective of overall oxidative stress (6). It is also likely that the concentrations of modified base and nucleoside in urine reflect the activities of different repair pathways responsible for the removal of 8-OH-Gua from DNA, i.e., base excision repair and nucleotide excision repair (NER) (7, 8), which produce, respectively, 8-OH-Gua and 8-OH-dGuo. The analysis of 8-OH-Gua in body fluids presents

particular difficulties (7, 9), but we have used HPLC followed by isotope-dilution gas chromatography–mass spectrometry to successfully measure 8-OH-dGuo and 8-OH-Gua in human urine (10, 11).

Cerebrospinal fluid (CSF) filters and disposes of degraded cellular biomolecules from the brain. The concentrations of these modified base/nucleosides in CSF should therefore reflect the involvement of the repair processes in the central nervous system, whereas their excretion in urine should represent the same phenomenon in tissues/organs throughout the organism (7, 12).

On the basis of experiments with cell culture, the hypothesis was put forward that, as opposed to other cell types, NER plays a major role in defending neurons from oxidative DNA damage (13). No in vivo data are available to support this concept, however. We therefore examined the amounts of 8-OH-dGuo and 8-OH-Gua excreted in urine and CSF. We hypothesized that the ratio of 8-OH-dGuo to 8-OH-Gua would be higher in CSF than in urine.

We studied 30 patients with histologically confirmed grade G2 (n = 17) and G3 (n = 13) primary intracranial tumors. Grade did not affect the concentrations of the modified base/nucleoside. Because smoking status may influence the excretion of modified base/nucleoside (7), we studied only patients with no history of smoking for at least 3 years. None of the patients had undergone chemotherapy during at least the 3 months before collection of urine and CSF. The group consisted of 16 men and 14 women (median age, 55 years; range, 42–83 years). We collected 24-h urine samples and 7 mL of lumbar CSF. The study was approved by the medical ethics committee of The Ludwik Rydygier Medical University (Bydgoszcz, Poland; No. 167/2001, in accordance with Good Clinical Practice, Warsaw 1998), and all patients gave informed consent.

We added 0.5 nmoles of [ $^{15}\text{N}_3, ^{13}\text{C}$ ]8-OH-Gua, 0.05 nmoles of [ $^{18}\text{O}$ ]8-OH-dGuo, and 10  $\mu\text{L}$  of acetic acid (HPLC grade; Sigma) to 2 mL of human urine. The isotopic purities of the internal standards were 97.65% and 85%, respectively. After centrifugation (2000g for 10 min), the supernatants were filtered through Millipore GV13 0.22  $\mu\text{m}$  syringe filters, and 500  $\mu\text{L}$  of each solution was injected in the HPLC system. In a pilot study, isotopically labeled internal standards of unmodified compounds (1 nmole of [ $^{13}\text{C}_3$ ]–Gua and 1 nmole of [ $^{15}\text{N}_5$ ]–dGuo) were added to the urine samples to monitor fractions containing both these compounds and to avoid overlapping peaks containing the modified and unmodified base/nucleoside. The isotopic purities of these internal standards were 96.4% and 98.0%, respectively.

We added 0.5 nmoles of [ $^{15}\text{N}_3, ^{13}\text{C}$ ]8-OH-Gua, 0.05 nmoles of [ $^{18}\text{O}$ ]8-OH-dGuo, and 10  $\mu\text{L}$  of acetic acid to 6 mL of CSF. After centrifugation (2000g for 10 min), the supernatants were dried by evaporation under reduced pressure in a Speed-Vac system, dissolved in 1 mL of water, and filtered through Millipore GV13 0.22  $\mu\text{m}$  syringe filters. We injected 500  $\mu\text{L}$  of each solution in the HPLC system.

HPLC purification of urinary 8-OH-Gua and 8-OH-dGuo was performed as described previously (10).

Gas chromatographic–mass spectrometric analysis (14) was adapted for analysis of [ $^{18}\text{O}$ ]8-OH-dGuo by monitoring the  $m/z$  442 and 457 ions.

We measured 8-OH-Gua and 8-OH-dGuo seven times on the same 1-mL urine samples to which 50 pmoles of 8-OH-Gua and 10 pmoles of 8-OH-dGuo had been added. The imprecision (CV) was 3.1% and 6.5% at 103 and 33 nmol/L, respectively, with mean (SD) recoveries of 97 (4)% and 94 (6)%.

The median [95% confidence interval (CI)] urinary concentrations of 8-OH-Gua and 8-OH-dGuo for the 30 patients were 121 (104–193) and 16 (14–21) nmol/L, respectively, whereas in CSF, the concentrations were 1.0 (1.1–2.3) and 0.7 (0.6–1.3) nmol/L, respectively.

Assuming a mean brain weight of 1.250 kg and a daily production of CSF of 576 mL [0.4 mL/min (15)], the median (95% CI) daily excretion of 8-OH-Gua and 8-OH-dGuo in CSF per kilogram of brain weight per 24 h was 0.48 (0.52–1.1) and 0.33 (0.27–0.62) nmol  $\cdot$  kg brain $^{-1}$   $\cdot$  24 h $^{-1}$ , respectively, and the daily excretion in urine was 2.66 (2.13–3.48) and 0.23 (0.20–0.29) nmol  $\cdot$  kg brain $^{-1}$   $\cdot$  24 h $^{-1}$ , respectively (Table 1).

Measurement of 8-OH-dGuo has been used to examine relationships between oxidative DNA damage and diseases (16–18). Deficient repair of oxidative DNA damage may contribute to the phenotype of Cockayne syndrome, an inherited disorder with a distinctive array of severe developmental and neurologic abnormalities. Cultured Cockayne syndrome cells are defective in transcription-coupled repair of oxidative DNA damage (19, 20). Accelerated repair of the lesion, especially in the template strand, is therefore presumably important for maintaining transcription of genes essential for cellular activity and neurologic functions. Recent studies have also demonstrated that the transcription-coupled repair mechanism can contribute to the repair of 8-OH-Gua in eukaryotes in vitro and in vivo (21, 22). Although there is no single protein involved in removal of 8-OH-dGuo via the NER pathway, 8-OH-dGuo appears to be a product of this repair mechanism [for a detailed discussion, see Ref. (8)]. One of the enzymes that may be involved in 8-OH-dGuo release via the NER pathway is an endonuclease reported by Bessho et al. (23). This protein can release 3',5',8-OH-dGDP (from modified DNA), which may subsequently be hydrolyzed to 8-OH-dGuo by nucleotidases (8). This

**Table 1. Calculated daily input of 8-OH-Gua and 8-OH-dGuo in CSF and urine of 30 patients.<sup>a</sup>**

|       | 8-OH-Gua, median<br>(95% CI) | 8-OH-dGuo, median<br>(95% CI) | 8-OH-Gua/8-OH-dGuo |
|-------|------------------------------|-------------------------------|--------------------|
| CSF   | 0.48 (0.52–1.1)              | 0.33 (0.27–0.62)              | 1.45:1             |
| Urine | 2.66 (2.13–3.48)             | 0.23 (0.20–0.29)              | 11.5:1             |

<sup>a</sup> Concentrations are in nmol  $\cdot$  kg $^{-1}$   $\cdot$  24 h $^{-1}$ . Concentrations in CSF are normalized to brain weight; concentrations in urine are normalized to body weight. These values assume a mean brain weight of 1.250 kg, a daily production of CSF of 576 mL (0.4 mL/min), and a body weight of 70 kg.

modified nucleoside in CSF and urine may therefore reflect the activity of NER in the central nervous system and whole body, respectively.

Our results support the thesis that the great majority of oxidatively damaged DNA is removed via the base excision repair pathway (24–26). However, the higher ratio of 8-OH-dGuo to 8-OH-Gua in CSF compared with urine suggests that NER may be particularly important in neurons (13).

Interestingly, the concentration of 8-OH-dGuo in CSF is approximately eightfold higher than plasma concentrations reported in the literature (12, 27). This high concentration may be attributable to a very high production rate in the brain and spinal cord, or it could result from slow transport from CSF through the blood–brain barrier. Because the consumption of oxygen by the brain is ~10-fold higher than in other organs (28, 29), our data are compatible with the interpretation that 8-OH-dGuo production is higher in the brain than in other tissues. The calculation of production from the concentration in CSF and an average reported production of CSF assumes, however, that the blood–brain barrier is easily permeable to 8-OH-dGuo and without active transport mechanisms for it. This remains to be verified before a final conclusion about the production of 8-OH-dGuo in the brain can be made.

We cannot exclude the possibility that mechanisms other than repair processes contribute to 8-OH-Gua and 8-OH-dGuo concentrations in human urine and CSF. Another possible source of 8-OH-dGuo may be that it derives from dead cells (30), or it could derive from sanitation of the cellular nucleotide pool by the MutT-directed pathway (31), and the excretion of 8-OH-Gua may also include a contribution from oxidized RNA.

This study was conducted with cancer patients because there is no justification for taking CSF from healthy individuals. However, the aim of the work was to compare the excretion of DNA repair products into CSF and urine. It is not known whether DNA repair mechanisms are different in cancer patients and healthy individuals. The mean concentrations of 8-OH-dGuo in CSF of healthy individuals and individuals with Alzheimer disease have been reported (32, 33) to be 684 and 196 nmol/L, respectively. These values are 100-fold higher than the concentrations that we found. Although the methods were different in the two studies, we think that a more important explanation for the different results is that the earlier study used postmortem CSF. It is possible that, after death, some degradation of cellular DNA increases the concentration of the modified nucleoside.

We conclude that the ratio of 8-OH-dGuo to 8-OH-Gua is approximately eightfold higher in CSF than in urine. This may reflect a more prominent role of NER in removal of oxidatively damaged DNA in the brain than in the average cell of the organism.

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**Effect of Anti-Carbonic Anhydrase Antibodies on Carbonic Anhydrases I and II**, Francesco Botrè,<sup>1\*</sup> Claudio Botrè,<sup>2</sup> Elisabetta Podestà,<sup>2</sup> Mauro Podda,<sup>3</sup> and Pietro Invernizzi<sup>3</sup> (<sup>1</sup> Controllo e Gestione delle Merci e del oro Impatto sull'Ambiente Department and <sup>2</sup> Department of Pharmacology and General Physiology, "La Sapienza" University of Rome, 00161 Rome, Italy; <sup>3</sup> Institute of Internal Medicine, Department of Medicine, Surgery and Dentistry, University of Milan, 20142 Milan, Italy; \* address correspondence to this author at: CGMIA Department, University of Rome "La Sapienza", Via del Castro Laurenziano 9, 00161 Rome, Italy; fax 39-06-23310228, e-mail botre@uniroma1.it)

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc enzyme that is widely distributed in the living world and is involved in many biochemical processes that depend on the hydration/dehydration of carbon dioxide/bicarbonate [reviewed in Refs. (1–6)].

Anti-CA antibodies have been identified, isolated, and purified from patients with a wide range of diseases, for some of which their presence can be a reliable diagnostic indicator (7). Anti-CA I and anti-CA II antibodies (aCAIab and aCAIIab) have recently been isolated from patients with systemic lupus erythematosus (8, 9), polymyositis and systemic sclerosis (9), endometriosis (10, 11), Sjögren syndrome (8, 9, 12, 13), idiopathic chronic pancreatitis (13, 14), primary biliary cirrhosis (PBC) (12, 13, 15, 16), and autoimmune cholangitis (15).

It has recently been hypothesized that all of these diseases (many of which can occur concomitantly) may have a common pathogenetic mechanism based on autoimmune reactions against a common antigen. According to this hypothesis, it seems that highly active CA isoenzymes (cytosolic CA II and membrane-bound CA IV) are particularly involved, because CA plays an important role in such biochemical processes as tissue hydration and secretory activities. In some cases, the preincubation of CA with specific inhibitors has blocked its antibody interactions, suggesting that the site of the immunologic reaction may involve the active site of the enzyme (9).

In patients with PBC, anti-CA antibodies are often associated with the presence of anti-mitochondrial anti-

bodies, particularly anti-pyruvate dehydrogenase (17–19), which is the main diagnostic marker of the disease (20, 21). However, anti-CA antibodies have also been detected in the absence of anti-mitochondrial antibodies in patients with PBC and ascites (16).

The aim of this study was to verify whether isoenzyme-specific anti-CA antibodies, isolated from patients with PBC and ascites, can affect the catalytic activity of the different CA isoenzymes and, if so, to evaluate the isoform and species specificity of the effect.

The experiments were carried out on samples of anti-CA antibodies purified by means of affinity chromatography. The catalytic activities of the purified human CA I (hCAI), human CA II (hCAII), and bovine CA II (bCAII) isoforms were tested in the presence and absence of anti-CA antibodies by use of an electrochemical method based on measuring the rate of CO<sub>2</sub> diffusion from a buffered NaHCO<sub>3</sub> solution (22).

Total IgGs were obtained by means of immunoabsorption through protein G columns (HiTrap Protein G column; no. 1-7001-00; Pharmacia Biotech Italia). Pure CA I and CA II IgGs were prepared by an immunoabsorption method in which purified human CA I and CA II antigens (Sigma Aldrich) were attached to *N*-hydroxysuccinimide (NHS)-activated columns (HiTrap NHS-activated column; no. 71-7006-00; Pharmacia Biotech Italia), and the specific IgGs were adsorbed by passing total IgG through the antigen-bound columns. The specific IgGs were then eluted from the affinity columns with 0.1 mol/L glycine-HCl buffer at pH 2.7. The mean spectrophotometrically assessed aCAIab concentration was 30 mg/L, and the mean aCAIIab concentration was 13 mg/L.

The catalytic activities of the different CA isoforms were assessed electrochemically in the presence and absence of chemical inhibitors and anti-CA antibodies (22, 23). The measurements were made by connecting a carbon dioxide microelectrode (MI 720 Microelectrodes Inc.) to a two-channel potentiometer (Orion 940 EA Ionalyzer; Analytical Control SpA); the electrode jacket was filled with an internal filling solution of NaHCO<sub>3</sub> (0.01 mol/L) and KCl (0.1 mol/L). All reagents were of analytical grade. The experiments were carried out in HEPES buffer, pH 7.0, in an open 200- $\mu$ L measuring cell that was maintained at 25 °C by means of forced water circulation and under magnetic stirring at a constant rate. The pH of the reaction chamber was monitored throughout the assay by a commercial pH glass microelectrode (MI 410; Microelectrodes Inc.). The CA activity correlates with the rate of CO<sub>2</sub> diffusion from the open chamber, as measured by the *P*CO<sub>2</sub> microelectrode (22). The potentiometric apparatus was calibrated with samples of crystalline CA I and II, and the measurements were made under the following experimental conditions:

For system calibration with hCAI, hCAII, and bCAII, CA activity was measured in 60  $\mu$ L of 0.1 mol/L HEPES, pH 7.0, at 25 °C. After a stable potential was reached, 20  $\mu$ L of substrate solution (0.1 mol/L NaHCO<sub>3</sub>) was added; subsequently, at the time of maximum electromotive force (E), a known volume of the enzymatic calibration solution