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Age and Organ Dependent Spontaneous Generation of Nuclear 8-Hydroxydeoxyguanosine in Male Fischer 344 Rats

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SUMMARY: 8-Hydroxydeoxyguanosine (8-OHdG) is a major oxidative DNA adduct playing roles in senescence, carcinogenesis and various disease processes. High-performance liquid chromatography with an electrochemical detection (HPLC-ECD) method has been widely used to assess organ levels of 8-OHdG, and a recently introduced immunohistochemical approach has made it possible to clarify intra-organ localization. In the present study, these methods were employed to reveal age-dependent changes in nuclear 8-OHdG within various tissues of male Fischer 344 rats between 18 fetal days and 104 weeks of age. 8-OHdG was detected in the nuclei of cerebellar small granule and small cortical cells, cerebral nerve cells, and choroid plexus epithelia of the brain and ependymal cells of the spinal cord; parenchymal cells in the anterior lobe of the pituitary and adrenal glands (mainly cortex); bronchial epithelium of the lung; intra-hepatic bile duct, pancreatic duct, glandular gastric and intestinal epithelial cells; renal tubular epithelial cells (mainly medulla); and spermatogonia and spermatocytes of the testis and seminal vesicle epithelia. The nuclear 8-OHdG levels were high (more than two lesions per 10^6 deoxyguanosines) from 7 days to 104 weeks of age in the brain, 3 to 6 weeks in the adrenal gland, 6 to 104 weeks in the lung, and 3 to 52 weeks in the testis. In the other organs, the nuclear 8-OHdG levels remained low throughout. These findings provide a basis for research dealing with oxidative stress by indicating organ-specific and age- but not aging-dependent changes in the localization of spontaneously generated nuclear 8-OHdG in intact rats. The immunohistochemical approach has advantages for assessing variation of 8-OHdG formation at the cellular level not accessible to the HPLC-ECD method. (*Lab Invest 2000, 80:249–261*).

Reactive oxygen species (ROS), damaging almost all classes of subcellular components, are produced in numerous pathophysiological states during the lives of aerobes, forcing these organisms to arm themselves with a variety of antioxidant defense systems. These include enzymatic decomposition of ROS, interruption of ROS-mediated reactions, and repair of ROS-induced damage, as well as subsequent alterations (eg, mutations due to oxidative DNA damage) (Ames et al, 1993; Anisimov, 1998; Harman, 1988). However, the defense systems may be overcome, leading to the phenomenon known as "oxidative stress" (Ames et al, 1993; Anisimov, 1998; Harman, 1988). In recent years, this has become recognized or suggested as participating in the development of senescence, carcinogenesis, and a wide variety of diseases, either aging-dependent or independent (Ames et al, 1993; Ando et al, 1998; Anisimov, 1998; Busciglio et al, 1998; Facchinetti et al, 1998; Feher et al, 1998; Feig et al, 1994; Floyd, 1990; Harman, 1988; Hasselwander and Young, 1998; Hogg, 1998; Kaplowitz and Tsukamoto, 1996; Kasai, 1997; Minamoto et al, 1999; Peterhans, 1997; Reiter, 1998; Saugstad, 1998; Schenker and Montalvo, 1998; Singal et al, 1998; Stadtman and Berlett, 1998; Thomson et al, 1998; Zs-Nagy et al, 1988).

Among over 30 different base modifications and other types of oxidative DNA damage (Bartsch, 1996; Halliwell and Aruoma, 1991; Nath et al, 1996; 1997), 8-hydroxydeoxyguanosine (8-OHdG) is the most abundant (Floyd, 1990; Halliwell and Aruoma, 1991; Kasai, 1997), resulting in mutations through formation of GC-to-TA transversions (Cheng et al, 1992; Kamiya et al, 1992; Moriya et al, 1991; Shibutani et al, 1991). Accumulation of 8-OHdG contributes to the mechanisms underlying the occurrence of "free-radical diseases" (Ames et al, 1993; Floyd, 1990; Kasai, 1997). Problems exist, however, in verifying the comparability of investigations dealing with 8-OHdG. Since its first discovery by Kasai and Nishimura's group (Kasai et al, 1984; Kasai and Nishimura, 1984), 8-OHdG levels have been determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD), gas chromatography with mass spectrometry, ^{32}P-postlabelling, or assays based on the use of repair endonucleases specific for the lesion, but good agreement has not been obtained among values

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given by different techniques (Collins et al, 1997). Furthermore, 8-OHdG levels appear to be altered in age- and organ-specific fashions, but again there is no good consensus among values (Fraga et al, 1990; Hirano et al, 1996; Kaneko et al, 1996; 1997; Randerath et al, 1997; Sai et al, 1992). Finally, while the conventional methods listed above are applicable for analysis of 8-OHdG levels on a whole organ basis, they do not allow for its localization within tissues. An immunohistochemical approach has been developed to solve this problem and is rapidly prevailing, using monoclonal antibodies specific for 8-hydroxyguanine moieties in DNA (Calderon-Garciduenas et al, 1999; Hayashi et al, 1999; Iihara et al, 1999; Kondo et al, 1999; Oberyszyn et al, 1998; Takahashi et al, 1998; Toyokuni et al, 1997; Won et al, 1999; Yarborough et al, 1996; Yoshida et al, 1999). The immunohistochemical data obtained thus far are still limited, and life-long studies to investigate the organ-specificity and aging-dependency of spontaneously generated 8-OHdG throughout the body of animals have not been conducted.

We have been exploring roles of 8-OHdG formation in the mechanisms underlying rat hepatocarcinogenesis but have encountered difficulties in determining sites of generation, especially in the presence and morphological changes such as fat accumulation, hepatocyte regeneration and deposition of non-epithelial components, including connective tissue (Nakae et al, 1990, 1994b, 1997a; 1997b). To overcome this and other problems facing investigators of 8-OHdG changes, accumulation of data from immunohistochemical studies of 8-OHdG localization is necessary. As a basis, however, steady-state data should first be obtained to avoid future confusion. We thus set the aim of the present study to assess spontaneous generation of nuclear 8-OHdG within various organs of intact rats from the prenatal period onwards.

Results

Age-Dependent Changes in Nuclear 8-OHdG Localization in Rat Organs

When positive for 8-OHdG immunohistochemistry, nuclei were stained almost homogeneously with fine granular signals. Larger positive granules were also occasionally detected in the cytoplasm, but their nature could not be elucidated.

There was no staining in any fetal organs (data not shown). The results for the 8-OHdG localization in positive organs of rats from 0 days to 104 weeks of age are summarized in Table 1. The heart and epididymis were negative throughout. In the cerebellum, small granule and small cortical cells were positive (Fig. 1A) in three and four out of five rats at 3 and 7 days of age, respectively, and in all animals aged 2 weeks or older. Whereas nerve cells in the cerebral cortex (Fig. 1B) had 8-OHdG only between 6 and 26 weeks, those in the brain stem (Fig. 1C) showed positive staining in one, three, four, three, and four out of five rats of 0, 1, 2, 3, and 7 days of age, respectively, and in all older animals. 8-OHdG was also detected in epithelial cells of the choroid plexus (Fig. 1D) and ependymal cells in the spinal cord (Fig. 1E) at 2 to 104 and 18 to 26 weeks of age, respectively. Parenchymal cells in the anterior but not the other lobes of the pituitary gland were stained positive (Fig. 2A) at 18 weeks of age or older. In the adrenals, 8-OHdG was detected only at 6 weeks of age in parenchymal cells of the medulla (Fig. 2B), whereas those in the cortex demonstrated positive signals (Fig. 2C) at 3 weeks of age and thereafter. Bronchial epithelial cells were stained positive at 6 weeks of age or older, but no signals were detected in the other components of the lung (Fig. 3). Intrahepatic bile duct (Fig. 4A) and pancreatic duct (Fig. 4B) cells were similarly 8-OHdG-positive between 6 and 104 weeks, while pancreatic islet cells gave positive signals only at 6 weeks of age. Hepatocytes, Kupffer cells, sinusoidal endothelial cells, and pancreatic acinar cells were all negative throughout (Fig. 4, A and B). 8-OHdG was detected in the glandular stomach and intestinal epithelia only in 6-week old rats. Glandular cells but only a few foveolar cells in the stomach (Fig. 4C) and cells present in the bottom of crypts (Fig. 4D) were stained positive. No signals were noted in the forestomach or esophagus (data not shown). Epithelial cells of the distal renal tubules were positive (Fig. 5A) in two, two, three, and three of five rats at 0, 1, 2, and 3 days of age, respectively, and in all five animals between 7 days and 26 weeks of age. 8-OHdG was detected in only the most distal cells at day 0, with the positive area gradually extending in the proximal direction. All of the renal medulla was positive after 7 days. In contrast, proximal tubules but not glomeruli were stained positive only at 6 weeks of age (Fig. 5B). 8-OHdG signals were demonstrated strongly in spermatogonia, weakly in spermatocytes and, sometimes, also weakly in sperm (Fig. 6A) in rats between 3 and 52 weeks of age but not at 104 weeks, when most of the testes were occupied by interstitial cell tumors obliterating the original architecture (data not shown). Seminal vesicles were stained positive between 3 and 52 weeks of age (Fig. 6B).

Age-Dependent Changes of Nuclear 8-OHdG Levels in Rat Organs

Certain criteria must be defined to judge quantitative alteration of 8-OHdG levels. It is not appropriate to use values at specific ages for each organ as control levels, but rather, data at all time points and for all organs should be taken into consideration and evaluated from a universal point of view. In the present study, therefore, a tentative normal upper-limit value was set at 2.00 ± 0.50 8-OHdGs per 10^6 deoxyguanosines (dGs), as this is the lowest average value for steady-state 8-OHdG levels cited in the literature, with only one exception (Collins et al, 1997; Helbock et al, 1999).

Table 2 summarizes data for the brain, adrenal gland, lung, liver, and testis. The nuclear 8-OHdG

Table 1. Age-dependent Changes in Immunohistochemically Detected Nuclear 8-OHdG Localization in Various Organs[a]

System	Organ	Cell type	Age (days old)					Age (weeks old) (5/5)[b]						
			0	1	2	3	7	2	3	6	18	26	52	104
Central nervous system	Cerebellum	Small granule cell	−	−	−	+ (3/5)	+ (4/5)	+	+	+	+	+	+	+
		Small cortical cell	−	−	−	+ (3/5)	+ (4/5)	+	+	+	+	+	+	+
	Cerebral cortex	Nerve cell	−	−	−	−	−	−	−	+	+	+	−	−
	Brain stem	Nerve cell	+ (1/5)[b]	+ (3/5)	+ (4/5)	+ (3/5)	+ (4/5)	+	+	+	+	+	−	−
	Choroid plexus	Epithelial cell	−	−	−	−	−	+	+	+	+	+	+	+
	Spinal cord	Ependymal cell	−	−	−	−	−	−	−	−	+	+	−	−
Endocrine system	Pituitary gland	Anterior lobal parenchymal cell	−	−	−	−	−	−	−	−	+	+	+	+
	Adrenal gland	Medullar parenchymal cell	−	−	−	−	−	−	−	+	−	−	−	−
		Cortical parenchymal cell	−	−	−	−	−	−	+	+	+	+	+	+
Respiratory system	Lung	Bronchial epithelial cell	−	−	−	−	−	−	−	+	+	+	+	+
Digestive system	Liver	Bile ductal epithelial cell	−	−	−	−	−	−	−	+	+	+	+	+
	Pancreas	Pancreatic ductal epithelial cell	−	−	−	−	−	−	−	+	+	+	+	+
		Islet cell	−	−	−	−	−	−	−	+	−	−	−	−
	Glandular stomach	Epithelial cell	−	−	−	−	−	−	−	+	−	−	−	−
	Intestine	Epithelial cell	−	−	−	−	−	−	−	+	−	−	−	−
Urinary system	Kidney	Distal tubular epithelial cell[c]	+ (2/5)	+ (2/5)	+ (3/5)	+ (3/5)	+ (5/5)	+	+	+	+	+	−	−
		Proximal tubular epithelial cell	−	−	−	−	−	−	−	+	−	−	−	−
Male genital system	Testis	Spermatogonium	−	−	−	−	−	−	+	+	+	+	+	−
		Spermatocyte	−	−	−	−	−	−	+	+	+	+	+	−
	Seminal vesicle	Epithelial cell	−	−	−	−	−	−	+	+	+	+	+	−

[a] No positive staining was detected in the fetus. Minus (−) and plus (+) symbols represent negative and positive staining, respectively.
[b] Data in parentheses are numbers of rats with positive 8–OHG staining per assessed rats.
[c] Only the most distal portion was positively stained at 0 days old, and the positive area extended gradually towards the proximal end.

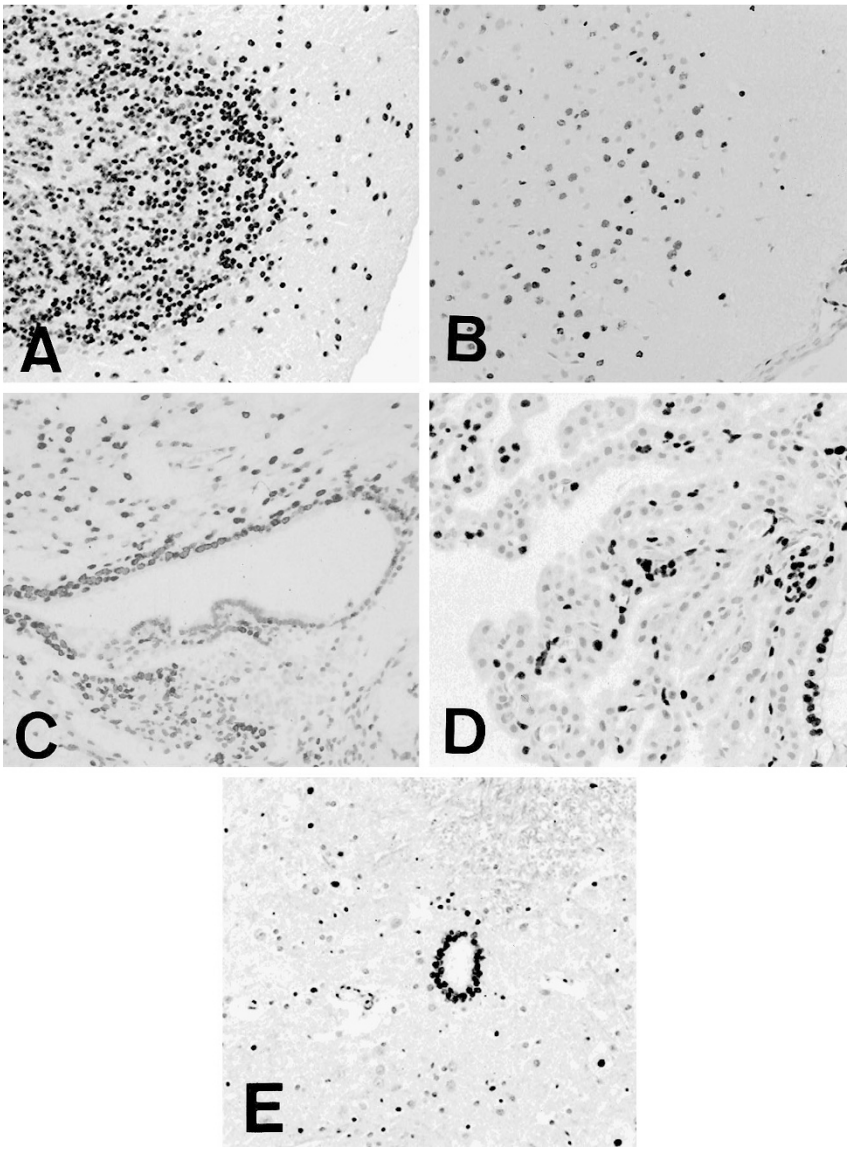

Figure 1.
Representative 8-OHdG immunohistochemistry in the central nervous system: Cerebellum at 26 weeks of age (A, ×66); cerebral cortex of a 6-week old rat (B, ×66); brain stem at 3 days (C, ×50); choroid plexus at 6 weeks (D, ×66); and spinal cord at 26 weeks (E, ×50).

levels in these organs became significantly greater than the upper-limit value at least once during the lifespan. In the brain, nuclear 8-OHdG levels significantly increased from 7 days of age, reached a plateau level at 2 weeks of age, and remained high (11.80–18.34 8-OHdGs per 10^6 dGs) until 104 weeks. In the adrenal gland, assessment could not be performed before 2 weeks of age because of the scarcity of tissue, but 8-OHdG levels were already high at 3 weeks. The values gradually decreased to below the upper-limit value at 18 weeks and remained low thereafter. In the lung, nuclear 8-OHdG levels increased at 6 weeks and then remained constant (8.35–12.05 8-OHdGs per 10^6 dGs). In the liver, nuclear 8-OHdG levels exceeded the upper-limit value only in 7-day old rats. In the testis, for which assays were not performed until 3 weeks of age because of the size problem, nuclear 8-OHdG levels were high until 52 weeks of age (5.12–8.15 8-OHdGs per 10^6 dGs), forming a peak value at 6 weeks (27.88 8-OHdGs per 10^6 dGs). No simple relationship with aging was noted for any organ. Nuclear 8-OHdG levels in the other assessed organs, including the pancreas, glandular stomach mucosa, colon mucosa, kidney, heart, and spleen, were below the upper-limit value throughout (Table 3).

Discussion

The present results demonstrate that nuclear 8-OHdG localization within organs and organ levels both change in an organ-specific manner and in an age-, but, unexpectedly, not aging-, dependent fashion. The immunohistochemical and HPLC-ECD results corresponded well in the cases of the lung, testis and heart, and a relatively good fit was also noted for the brain,

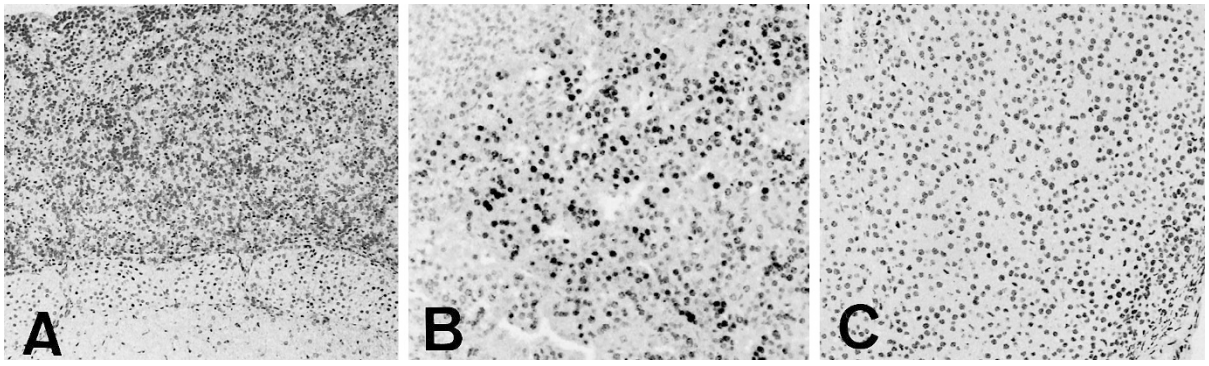

Figure 2.
Representative 8-OHdG immunohistochemistry in the endocrine system: Pituitary gland of an 18-week old rat (A, ×33); adrenal medulla at 6 weeks (B, ×50); and adrenal cortex at 3 weeks (C, ×66).

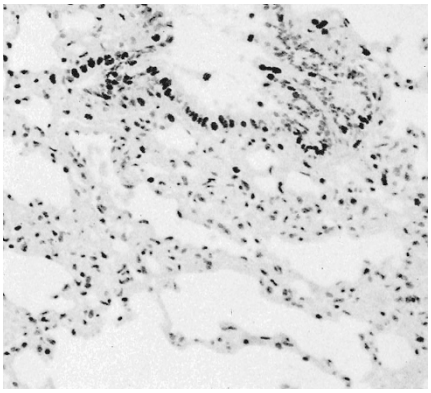

Figure 3.
Representative 8-OHdG immunohistochemistry in the respiratory system: Lung tissue of an 18-week old rat (×50).

glandular stomach and colon. In contrast, good accordance was lacking for the adrenal gland, liver, pancreas and kidney. Whereas 8-OHdGs were localized in a majority of cells in the organs with a good fit, with the exception of the lung, when the lesion levels were high, they were detected in only minor populations in those lacking a good fit. In the lung, large amounts of 8-OHdG appeared to be produced in bronchial epithelia between 6 and 104 weeks of age. The present results thus indicate the utility of the immunohistochemical approach, allowing exact localization and detection of 8-OHdG when the lesions are too few to be picked up by conventional techniques on a whole-organ basis.

The overall ROS production increases, while the overall efficiency of the antioxidant defense systems decreases during aging (Ames et al, 1993; Martin et al, 1996; Reiter et al, 1995; Shigenaga et al, 1994), with a clear organ-specificity (Sohal et al, 1995; Tian et al, 1998). These are factors determining 8-OHdG levels at a particular age in a particular organ, and the other factors include the status of repair mechanisms, oxygen exposure, metabolic and oxygen consumption rates, and cell proliferation activity (Ames et al, 1993; Fraga et al, 1990). It is thus noteworthy that most of the assessed organs demonstrated 8-OHdG signals at 6 weeks, when growth is marked in the young adult. In the cases of the brain and testis, the status of the blood barrier (Fraga et al, 1990), preventing these organs from exposure to contaminating xenobiotics and endogenously produced substances, might also be a factor.

With regard to the pre- and postnatal changes in 8-OHdG steady-state levels in rat organs, Randerath et al (1997) demonstrated liver increase within 1 hour after birth, a peak at the 53rd hour and decrease by the 100th hour in neonatal Fischer 344 rats. We could not detect any elevation of nuclear 8-OHdG levels in the assessed organs during the prenatal and postnatal/preweaning period up to 2 weeks of age using HPLC-ECD, with the single exception of a small increase in the liver at 7 days. However, we could immunohistochemically detect 8-OHdG in the brain stem and kidney, but not in the liver, immediately after birth. The data thus indicate that rats are indeed confronted with early-postnatal oxidative stress (Gunther et al, 1993; Krukowski and Smith, 1976; Yoshimura et al, 1988). The discrepancy between our findings and those of Randerath et al (1997) may be, at least partly, due to the differences in detection methods (they apply ^{32}P-postlabeling) and the possible presence of mitochondrial DNA contamination.

Fraga et al (1990) reported aging-dependent increase of 8-OHdG in the liver, kidney and intestine, but not brain or testis, of male Fischer 344 rats from 1 to 24 months of age. Sai et al (1992) described lesion levels in the same strain that increased significantly in the liver and kidney of both sexes, slightly in the lung and spleen of females, but not in the brain of either sex, in an aging-dependent fashion between 6 and 30 months of age. However, Hirano et al (1996) subsequently re-assessed this issue using male Sprague-Dawley rats and demonstrated no apparent change in 8-OHdG levels in the brain, lung, liver, small intestine, kidney or spleen between 3-week and 30-month old animals. Kaneko et al (1996; 1997) confirmed these results in the brain, liver, kidney or heart of male Fischer 344 rats between 2 and 24 months of age but showed an increase in lesion levels from 24 to 33 months. While all four of these studies employed the HPLC-ECD method, discrepancies may well be ex-

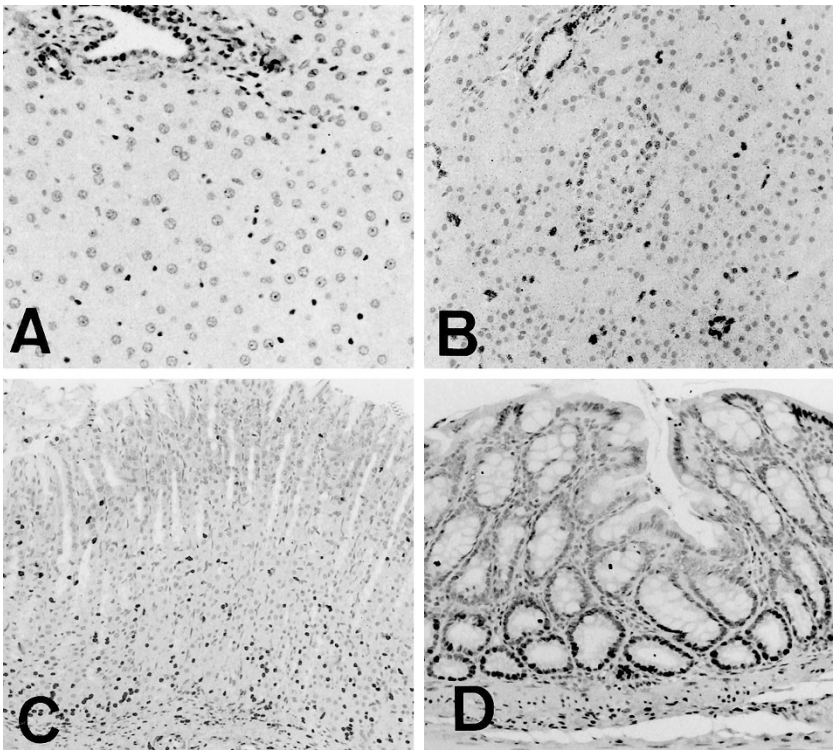

Figure 4.
Representative 8-OHdG immunohistochemistry in the digestive system: Liver at 18 weeks (A, ×50); pancreas at 6 weeks (B, ×66); glandular stomach at 6 weeks (C, ×40); and intestine at 6 weeks (D, ×40).

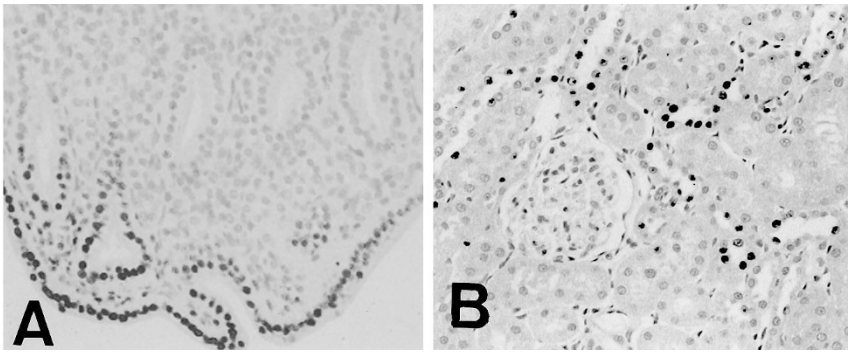

Figure 5.
Representative 8-OHdG immunohistochemistry in the urinary system: Renal medulla of a 2-day old rat (A, ×50); and renal cortex at 6 weeks (B, ×80).

plained by the fact that contamination of mitochondrial DNA was not always avoided (Hirano et al, 1996). In our study, the brain levels were high (12.22–18.34 8-OHdGs per 10^6 dGs), whereas those in the other assessed organs, except the adrenal gland, lung and testis, were low (0.40–1.88 8-OHdGs per 10^6 dGs) throughout the postweaning lifespan (3 weeks of age or older). Although a tendency for elevated 8-OHdG levels in the brain was seen in the work of Hirano et al (1996), it was not clear, and "low" levels in the preceding studies were always around a single 8-OHdG within 10^5 dGs, corresponding to "high" in the present study. Substantial decline of background noises can be achieved by improving the HPLC-ECD method with introduction of a reliable DNA extraction using a commercially available kit (Helbock et al, 1998; 1999; Nakae et al, 1995).

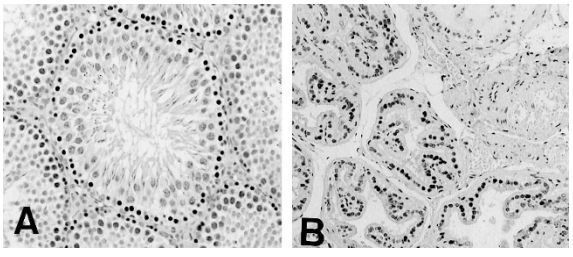

Figure 6.
Representative 8-OHdG immunohistochemistry in the male genital system: Testis at 6 weeks (A, ×33); and seminal vesicle at 26 weeks (B, ×50).

Table 2. Age-dependent Changes in Nuclear 8-OHdG Levels in the Brain, Adrenal Gland, Liver, and Testis

	8-OHdG levels (8-OHdG/10^6 dG) Organ				
Age	Brain	Adrenal gland	Lung	Liver	Testis
18 fetal days old	1.89 ± 0.08[a]	Not assessed	0.71 ± 0.16	1.53 ± 0.09	Not assessed
0 day old	1.08 ± 0.34	Not assessed	1.18 ± 0.58	1.20 ± 0.12	Not assessed
1 day old	1.58 ± 0.07	Not assessed	1.33 ± 0.27	1.18 ± 0.25	Not assessed
2 days old	0.65 ± 0.05	Not assessed	0.91 ± 0.42	1.55 ± 0.43	Not assessed
3 days old	1.88 ± 0.06	Not assessed	0.67 ± 0.14	1.27 ± 0.18	Not assessed
7 days old	5.89 ± 0.21[b]	Not assessed	0.38 ± 0.11	3.95 ± 0.26[c]	Not assessed
2 weeks old	11.80 ± 1.26[c]	Not assessed	0.39 ± 0.07	1.43 ± 0.40	Not assessed
3 weeks old	14.22 ± 1.67[c]	8.28 ± 1.74[c]	0.56 ± 0.13	0.93 ± 0.36	8.15 ± 1.26[c]
6 weeks old	12.22 ± 2.23[c]	3.62 ± 0.48[b]	8.35 ± 0.83[c]	0.85 ± 0.15	27.88 ± 3.85[c]
18 weeks old	15.06 ± 2.09[c]	1.25 ± 0.26	11.02 ± 2.31[c]	0.75 ± 0.16	7.15 ± 1.02[c]
26 weeks old	18.34 ± 4.91[c]	0.96 ± 0.55	12.05 ± 2.31[c]	0.64 ± 0.07	4.86 ± 0.42[b]
52 weeks old	16.22 ± 1.57[c]	0.73 ± 0.25	8.53 ± 1.63[c]	0.90 ± 0.26	5.12 ± 0.78[b]
104 weeks old	16.53 ± 3.20[c]	0.75 ± 0.27	10.23 ± 1.14[c]	0.99 ± 0.43	0.72 ± 0.03

[a] Data are means ± standard deviations of values obtained from 5 independent animals.
Significantly more than the tentative upper–limit value of 2.00 ± 0.50 ($p < 0.05^b$ or 0.01^c).

Table 3. Age-dependent Changes in Nuclear 8-OHdG Levels Detected in the Pancreas, Stomach, Colon, Kidney, Heart, and Spleen

	8-OHdG levels (8-OHdG/10^6 dG) Organ					
Age	Pancreas	Stomach[a]	Colon[a]	Kidney	Heart	Spleen
18 fetal days old	Not assessed	Not assessed	Not assessed	0.62 ± 0.32[b]	Not assessed	Not assessed
0 day old	Not assessed	Not assessed	Not assessed	0.87 ± 0.15	Not assessed	Not assessed
1 day old	Not assessed	Not assessed	Not assessed	0.72 ± 0.27	Not assessed	Not assessed
2 days old	Not assessed	Not assessed	Not assessed	0.65 ± 0.27	Not assessed	Not assessed
3 days old	Not assessed	Not assessed	Not assessed	0.92 ± 0.54	Not assessed	Not assessed
7 days old	Not assessed	Not assessed	Not assessed	1.32 ± 0.30	Not assessed	Not assessed
2 weeks old	Not assessed	Not assessed	Not assessed	1.31 ± 0.29	Not assessed	Not assessed
3 weeks old	0.60 ± 0.36	0.62 ± 0.03	0.99 ± 0.41	0.72 ± 0.34	0.71 ± 0.39	0.40 ± 0.12
6 weeks old	0.62 ± 0.25	0.62 ± 0.27	0.79 ± 0.40	1.15 ± 0.32	0.71 ± 0.17	1.12 ± 0.13
18 weeks old	0.65 ± 0.22	0.48 ± 0.14	0.67 ± 0.27	1.02 ± 0.44	0.92 ± 0.14	0.68 ± 0.10
26 weeks old	0.73 ± 0.26	0.48 ± 0.18	0.55 ± 0.28	1.19 ± 0.34	0.80 ± 0.37	0.50 ± 0.09
52 weeks old	0.67 ± 0.27	0.51 ± 0.31	0.75 ± 0.41	0.72 ± 0.31	0.70 ± 0.17	1.88 ± 0.65
104 weeks old	0.59 ± 0.19	0.42 ± 0.14	0.70 ± 0.22	0.91 ± 0.53	0.81 ± 0.22	1.55 ± 0.27

[a] Mucosa only.
[b] Data are means ± standard deviations of values obtained from 5 independent animals.

The accumulation with age of spontaneous mutations has long been considered to play key roles in senescence (Vijg et al, 1997; Vijg and van Steeg, 1998; Walter et al, 1997). It is of interest, therefore, whether some relationships could be obtained between spontaneously generated mutations and 8-OHdGs in nuclear DNA. Spontaneous mutations in nuclear DNA indeed accumulate with age, but recent research using a variety of transgenic mice has revealed that such an accumulation occurs non-linearly and in an organ-specific fashion (Gossen and Vijg, 1993; Martus et al, 1995; Vijg et al, 1997; Vijg and van Steeg, 1998). Thirty to 40% of the mutations are detected in the brain and kidney, whereas 10–20% are in the liver, lung and spleen (Boerrigter, 1998). About one third of such spontaneous mutations arise before birth, about another third during the growth period of youth, and the remainder during the rest of the animal's life (Paashuis-Lew and Heddle, 1998). Mutation frequencies in the liver increase with aging, while those in the brain are not altered with age (Dollé et al, 1997). Spontaneous mutations are detected substantially less in male germ cells than in somatic cells (Gossen and Vijg, 1993). Among male germ cells, mutation frequencies are relatively high in primitive type A and type A spermatogonia, dramatically decreased in type B spermatogonia, and remain this low in more matured forms of spermatogenic cells in 60-day (8.5 weeks) and 80-week old mice, but such a decrease is not seen in 196-week old animals (Walter et al, 1998). Despite the presence of some similarities, such as the cell-type specificity demonstrated in the testis, there-

fore, there are obvious differences between mutations and 8-OHdGs, both spontaneously generated in nuclear DNA, in terms of the organ-specificity and the age-dependency. The simplest reason for such differences is the fact that 8-OHdG is pro-mutagenic but requires DNA replication without being repaired to cause mutation (Cheng et al, 1992; Kamiya et al, 1992; Moriya et al, 1991; Shibutani et al, 1991). Thus, the presence of 8-OHdG does not necessarily indicate that of mutation. Furthermore, the above-mentioned mutation research was all done in mice, while the present study was conducted in rats. Spontaneous mutation frequencies in the liver of recently developed transgenic rats are substantially lower than those of the transgenic mice similarly developed, and mutation patterns induced by aflatoxin B_1 are clearly different in these two animals (de Boer et al, 1996; Dycaico et al, 1996). It is thus apparent that the mutating events, either spontaneous or induced, are species-dependent, but the available data for species other than mice are thus far limited. In addition, GC-to-AT transitions are the most frequent class among spontaneous mutations (de Boer et al, 1996; Dycaico et al, 1996; Gossen and Vijg, 1993; Kohler et al, 1991; Martus et al, 1995; Vijg et al, 1997; Vijg and van Steeg, 1998), while 8-OHdG specifically induces GC-to-TA transversions (Cheng et al, 1992; Kamiya et al, 1992; Moriya et al, 1991; Shibutani et al, 1991). Different forms of oxidative DNA damage, however, can induce different types of mutations, including transitions, deletions, and frameshifts (Gille et al, 1994; Hsie et al, 1986; Jackson et al, 1998, Kreutzer and Essigman, 1998).

Nuclear 8-OHdG formation indicates the presence of oxidative stress in the nuclei, which can induce various epigenetic changes (Anisimov, 1998). These then alter the expression of specific genes, largely influence a variety of signal transduction pathways responsible for the maintenance and regulation of cellular functions, and in turn cause a variety of disorders (Anisimov, 1998; Finkel, 1999; Gameley and Kiyubin, 1999; Hampton et al, 1998; Hogg, 1998). The hypomethylation of specific genes is a particular example of such epigenetic changes, because ROS are among its main mediators (Cerda and Weitzman, 1997), while the presence of 8-OHdG per se inhibits DNA methylation (Turk et al, 1995; Weitzman et al, 1994). The hypomethylation occurs in an organ- and gene-specific and age-dependent manner and has been shown to play one of the central roles in both senescence and carcinogenesis (Anisimov, 1998; Christman, 1995; Poirier, 1994). A unique gene has recently been identified to be involved in the suppression of multiple aging phenotypes (Aizawa et al, 1998; Kuro-o et al, 1997; Matsumura et al, 1998). This gene, *klotho*, might be a candidate target of epigenetic influences due to nuclear oxidative stress because its expression is down-regulated under situations such as diabetes mellitus, chronic renal failure (Aizawa et al, 1998), and lipopolysaccharide-induced inflammation (Ohyama et al, 1998), in all of which oxidative stress plays a critical role (Finkel, 1999; Hasselwander and Young, 1998; Hogg, 1998; Peterhans, 1997; Singal et al, 1998). The mitochondrion is a major cellular source of ROS, and thus its components are immediate targets of oxidative stress. Mitochondrial DNA is much more susceptible to oxidative injury than nuclear DNA (Richiter et al, 1988), presumably because of its attachment to the inner mitochondrial membrane, a place where ROS are mainly generated, its lack of histones, and the limited capability of its repair systems (Ames and Gold, 1991; Clayton et al, 1974; Linnane et al, 1989; Miquel, 1991; Wallace, 1992). Incidentally, mitochondrial DNA mutates at a faster rate than nuclear DNA (Brown et al, 1979 and 1982). In mitochondria, respiratory activity decreases (Walter et al, 1997), and ROS generation increases (Sohal et al, 1995) with age. The oxidative submitochondrial damage and mutations thus lead to a variety of dysfunctions in the organella and also in whole cells, which in turn cause aging-dependent compromise of the neuronal, neuroendocrine, and immune systems (Shigenaga et al, 1994; Walter et al, 1997). Mecocci et al (1993) reported extensive aging-dependent increase of 8-OHdG levels in mitochondrial DNA with very little change of the lesion levels in nuclear DNA in the brain tissues of 10 control patients who died between the ages of 42 and 97 years and did not have neurological disease. Hayakawa et al (1991 and 1992) also reported an aging-dependent accumulation of 8-OHdG and mutations of mitochondrial DNA of human heart and diaphragm tissues. However, oxidative damage of cellular protein increases with age in the brain and liver of rats (Tian et al, 1998). In contrast, while the extent of lipid peroxidation in the brain or liver of rats is not altered by aging (Lopez-Torres et al, 1992; Tian et al, 1998), its byproducts can react with DNA to form dG malondialdehyde adducts, of which frequency increases in the liver and kidney but not in the testis with age in rats (Draper et al, 1995).

The organ-specific and age-dependent changes of nuclear 8-OHdG localization within rat tissues and the organ levels of the lesion should serve as a base for studies of the roles of this type of oxidative DNA damage in disease processes as well as for the risk assessment of environmental chemicals. As was discussed, however, further studies are apparently demanded to elucidate the detailed roles of nuclear and extra-nuclear oxidative stress in the processes of senescence, carcinogenesis, and a wide variety of diseases, in association with research about its relationships with various genetic and epigenetic changes.

Materials and Methods

Animals

Pregnant nulliparous Fischer 344 rats were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan) on the 12th or 13th day of gestation. On the 18th day of gestation, 10 fetuses were obtained by Caesarian section from dams under light ether anesthesia. From the other dams, 60 male litters were obtained and

sacrificed under light ether anesthesia, 10 each at 0, 1, 2, 3, and 7 days and 2 weeks postnatally. The whole bodies of half of these fetuses and serially sacrificed neonates were fixed in 10% neutrally buffered formalin, after the furs were removed with nicks in the thoracic and abdominal walls and skulls. Appropriate numbers of sagittal slices were made through the animal bodies after 1 week, and these were further fixed for another day before routine processing and embedding in paraffin. Serial 4-μm-thick specimens on silane-coated slide glasses were prepared for assessment of tissue distribution. From the other half of the fetuses and neonates, brains, lungs, livers and kidneys were excised, immediately frozen in liquid nitrogen and stored at −80° C until use. A total of 35 male Fischer 344 rats were also obtained just after weaning from Japan SLC. Ten of them (five each for immunohistochemistry and for the HPLC-ECD assay) were acclimatized until they reached 3 weeks of age and then sacrificed by exsanguination from the abdominal aorta under light anesthesia. The remaining 25 were maintained for scheduled sacrifice of five rats each at the ages of 6, 18, 26, 52, and 104 weeks. Brains, pituitary glands, abdominal parts of the spinal cord, adrenal glands, lungs, livers, pancreases, gastrointestinal tracts, kidneys, testes with epididymides and seminal vesicles, and hearts were excised. Spinal cords, epididymides, seminal vesicles, and appropriate parts of the other organs were fixed in 10% neutrally buffered formalin for 24 hours, embedded in paraffin and made into 4-μm-thick specimens on silane-coated slide glasses. The remaining parts of the organs were immediately frozen in liquid nitrogen and stored at −80° C until use. Only separated mucosae were frozen in the cases of the glandular stomach and colon.

Rats were housed in plastic cages with white flake bedding (Kansai Animal Corporation, Kyoto, Japan) in an air-conditioned (10–15 ventilations per hour) atmosphere, with a constant temperature (25 ± 3° C) and relative humidity (55 ± 8%), and a 12-hour dark/light cycle. Free access to a CE-2 diet (Crea Japan, Inc., Meguro, Tokyo, Japan) and tap water was always guaranteed. Body weights and consumption of food and tap water were monitored weekly until 10 weeks of age and biweekly thereafter.

8-OHdG Immunohistochemistry

Two different monoclonal antibodies were used. One of them (Yarborough et al, 1996) was generously supplied by Dr. Regina M. Santella (Columbia University, New York, New York). The other was purchased from the Japan Institute for the Control of Aging (Fukuroi, Shizuoka, Japan). While both antibodies generated closely similar immunohistochemical findings in the preliminary study, the commercial one was used in the main study due to its general availability. After deparaffinization, specimens were antigen-retrieved by autoclaving at 121° C for 15 minutes in a 10% zinc sulfate solution and then treated with 2 M HCl at 37° C for 30 minutes. After washing two times in 10 mM Tris-HCl buffer, pH 7.0, containing 0.88% NaCl and 0.25% Triton X-100 (Sigma Chemical Company, St. Louis, Missouri) (TTBS buffer) for 10 minutes, blocking with TTBS buffer containing 10% horse serum (Dako A/S, Copenhagen, Denmark) was performed at room temperature for 5 minutes. The specimens were then exposed to either of the two primary antibodies diluted 500-fold in TTBS buffer containing 10% horse serum at 4° C overnight. After washing as above, secondary antibody (within a LSAB2/HRP kit, Dako) treatment at room temperature for 10 minutes, re-washing, and removal of nonspecific binding by 70% methanol containing 0.3% hydrogen peroxide at room temperature for 30 minutes were sequentially performed. The specimens were then washed again, and the standard avidin-biotin complex procedure with a LSAB/HRP kit was conducted. After re-washing, binding was visualized with 25 mM Tris-HCl buffer, pH 7.4, containing 0.05% diaminobenzidine (Sigma) at room temperature for less than 10 minutes. Finally, counterstaining was performed with hematoxylin. The specificity of the antibodies to 8-hydroxyguanine moieties in DNA (8-OHdG after the DNA denaturation) and the identity of immunohistochemically detected signals as 8-OHdG had previously been confirmed (Takahashi et al, 1998; Toyokuni et al, 1997; Yarborough et al, 1996).

HPLC-ECD Assay for Nuclear 8-OHdG Levels

The 8-OHdG levels in nuclear DNA were measured using samples of organs (tissues) stored at −80° C. Portions weighing 50–100 mg were pulverized in liquid nitrogen, and DNA was extracted by our chaotropic NaI isolation method using a DNA Extractor WB kit (Wako Pure Chemical Industries, Limited, Kyoto, Japan) (Nakae et al, 1995) with a slight modification (Helbock et al, 1998 and 1999). DNA hydrolysis and microfiltration of the resultant samples were subsequently conducted by the method of Helbock et al (1999). The levels of 8-OHdG were then determined by an adaptation of the HPLC-ECD method of Kasai et al (1987) as described elsewhere (Nakae et al, 1994a; Yoshiji et al, 1992). Peaks gained with electrochemical (for 8-OHdG) and UV (for dG) detectors were integrated with a background noise correction loaded on an integrator. Values for 8-OHdGs per 10^6 dGs were obtained by calibration against curves from runs of standard samples, containing known amounts of authentic 8-OHdG (Wako) and dG (Sigma). During the assays, light and air contamination were avoided as strictly as possible. The completeness of the DNA hydrolysis (Kuchino et al, 1987) and the identity of the ECD peak as 8-OHdG (Nakae et al, 1997a) with the current procedures have been confirmed.

Statistics

Statistical analyses were performed using a personal computer, Power Macintosh G3 B/W 300 MHz (Apple Computer, Inc., Cupertino, California) with Mac OS System 8.5.1J (Apple) as the operation system and

InStat for Macintosh 2.0.3E (GraphPad Software, Incorporated, San Diego, California) as the statistical software. One-way analysis of variance was performed to determine variation among the group means. This was followed by Bartlett's test to determine the homogeneity of variance. To assess the statistical significance of an increase of the data from the tentative normal upper-limit value of 2.00 $\pm$ 0.50 8-OHdGs per 10^6 dGs (see *Age-dependent Changes of Nuclear 8-OHdG Levels in Rat Organs* in Results), the Dunnet multiple comparisons test was applied.

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