

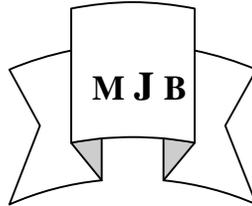
Immunohistochemical Detection of MDA –DNA Adducts in Oral Mucosa of Smokers and Nonsmokers

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Abstract

Malondialdehyde (MDA) is a major lipid peroxidation product that is mutagenic and tumorigenic. MDA-modified DNA adducts have been detected in animal and human tissues and may be a marker of human cancer risk. An immunohistochemical method, using a previously generated monoclonal antibody specific for MDA–DNA adducts, has been developed for the detection and quantification of DNA damage in human oral mucosa cells. The method was used initially on mice liver cells treated with and without MDA, and then applied to the detection of adducts in oral mucosa cells of smokers and non-smokers. Levels of DNA damage were elevated in 25 smokers (mean relative staining intensity 97 ± 41) compared with 25 age-, and sex-matched non-smokers (74 ± 17 , $P < 0.02$). These results demonstrate that MDA–DNA adducts can be measured in single cells of human samples by an immunohistochemical method. This methodology provides a simple way to monitor MDA–DNA damage and should be useful for studies investigating the role of exogenous and endogenous agents in oxidative stress and carcinogenesis.

الخلاصة

يعتبر مالونديالدهيد (إم دي أي) كأعظم مُنتَج رئيسي للبيروكسيد الشحمي الذي يؤدي إلى الطفرة الوراثية الجينية والتغيرات الورمية. إن عامل إم دي أي المُعدَّل دي إن أي قد تم الكشف عنه في الأنسجة الحيوانية والإنسانية وقد يكون علامة خطرٍ للسرطان الإنساني. طريقة الامينوبيروكسيد المناعية باستعمال أجسام مضادة أحادية السلسلة معينة سابقاً لعامل إم دي أي دي إن أي، تم تطويرها للكشف وقياس الضرر ل(دي إن أي) في خلايا الغشاء المخاطي الإنسانية. استعملت هذه الطريقة أولاً على خلايا كبد الفئران التي عولجت مع وبدون إم دي أي، وبعد ذلك استخدمت للكشف عن هذه العوامل في خلايا الغشاء المخاطي للغم في المدخنين والغير المدخنين. أظهرت النتائج إن مستويات الضرر ل(دي إن أي) قد ارتفعت في 25 مدخن (معدل الكثافة للصبغة 97 ± 41) وقورنت ب 25 حالة سيطرة من نفس العمر والجنس، من غير المدخنين (74 ± 17 ، بي > 0.02). هذه النتائج تُبين بأن عامل إم دي أي دي إن أي يُمكن أن يُقاس في الخلايا المفردة من العينات الإنسانية بطريقة الامينوبيروكسيد المناعية. هذه الطريقة المنهجية تُزوّدنا بطريقة بسيطة لمراقبة إضرار إم دي أي دي إن أي ويجب أن تكون مفيدة للدراسات المستخدمة للتحرّي عن دور العوامل الخارجية والذاتية في إلهاد الاوكسجيني والنشوء السرطاني.

Introduction

Malondialdehyde (MDA) is a naturally occurring endogenous product of lipid peroxidation and prostaglandin biosynthesis; it participates in a variety of chemical and biological reactions including covalent binding to protein, RNA and DNA [1]. The reaction of MDA with

deoxyguanosine gives rise to cyclic adducts, formed by the reaction of the carbonyl carbons of MDA at the N₁ and N₂ position of guanine. The major DNA adduct of MDA is a pyrimidopurine of deoxyguanosine (M1G) [2], although adenine adducts are also formed [3]. MDA is mutagenic in bacterial and mammalian

cells producing both frame shift and base pair mutations [4], and is carcinogenic in rats [2].

The endogenous formation of MDA during intracellular oxidative stress and its reaction with biologically important macromolecules makes MDA-DNA adducts a suitable biomarker of endogenous DNA damage. Marnett *et al.* have developed sensitive and reliable analytical methods for the detection and quantification of adducts in human and animal samples, including gas chromatography/mass spectroscopy (GC/MS) [5–8] and ^{32}P -post-labeling [9]. More recently, a monoclonal antibody was developed against M1G coupled to a carrier protein and found to have low cross-reactivity with other related endogenous exocyclic adducts [1]. When used in an immunoslot blot assay, the detection level was 2.5 adducts/ 10^8 nucleotides, comparable with that of other methods, but requiring much lower amounts of DNA [10]. The immunoassay was validated by the comparison of data on four white blood cell DNA samples to adduct detection by high-performance lipid chromatography (HPLC)/post-labeling [10]. After correction for the 50% recovery of the HPLC/post-labeling method, there was a good correlation between the two assays with slightly lower levels reported for the immunoassay. MDA-DNA adducts have been detected in human liver and pancreas by GC/MS [6,7] and in breast, lung and white blood cells by post-labeling [9–12]. Using these methods, levels of MDA-dG in various human tissues ranged from 2.2 adducts/ 10^9 to 1.1 adducts/ 10^6 nucleotides by the different methods [6,7,9–12].

Immunohistochemical methods have been used extensively to monitor DNA damage in humans with exposure to polycyclic aromatic hydrocarbons, aflatoxin B₁, 4-aminobiphenyl, 8-methoxypsoralen and UV radiation [13]. Oxidative DNA damage has also been measured in human tissues [14]. Here, we have used the monoclonal antibody against MDA-DNA to develop an

immunoperoxidase method for detection of this damage. The method was initially tested using cells treated with MDA, then applied to the detection of damage in oral mucosa cells of smokers and non-smokers. This method should be useful for the sensitive detection of DNA damage in small numbers of cells and tissue sections.

Materials and Methods

To develop the quantitative immunoperoxidase method, animal liver cells were cultured in 8 chambered slides with 10% fetal calf serum with L-glutamine. When cells were 50–60% confluent, they were treated for 2.5 h at 37°C with 0, 10, 20 and 40 mM MDA which was prepared from malonaldehyde bis(diethyl acetal) by treatment with acid as described previously [10]. After treatment, cells were washed with 1x phosphate-buffered saline (PBS) twice and fixed in –20°C methanol: acetic acid (3:1) for 10 min, then transferred to acetone for another 10 min.

Volunteers were recruited. After informed consent was obtained, oral mucosa cells were collected by rinsing the mouth with 1x PBS. Cells were smeared on pre-coated slides, air-dried and fixed. Samples were obtained from 25 smokers of at least 1 pack/day (range 20–30 cigarettes/day) and 25 race-, sex- and age-matched (within 5 years) non-smokers and were coded for analysis. Information on smoking history was collecting.

Immunoperoxidase staining

For immunoperoxidase staining, fixed slides of human oral mucosa cells and mice liver cells were washed with 1x PBS, treated with RNase (100 µl/ml; Sigma Chemical) at 37°C for 1 h, washed with 1x PBS, treated with proteinase K (10 µg/ml; Sigma) at room temperature for 10min and washed. To denature the DNA, slides were incubated with 4 N HCl for 10 min and then with 50 mM Tris base for 5 min, both at room temperature. After washing with 1x PBS, slides were incubated with 0.3% H₂O₂ in methanol at room temperature for 30 min

to quench endogenous peroxidase activity. Non-specific binding was blocked with 1.5% normal horse serum and then slides were incubated overnight at 4°C with anti-MDA monoclonal antiserum number D10A1, 1:6000 dilution in 1.5% normal horse serum). Elite mouse ABC and DAB kits (Vector Laboratories) were used for visualization of bound antibody as directed by the manufacturer. Slides were dehydrated and cleaned in serial ethyl alcohol and xylene and mounted with Permount. To demonstrate the specificity of staining, human oral cells from a smoker were pre-treated with DNase (500 µg/ml for 1 h at 37°C) before staining, stained with a non-specific antibody (9D8, 1:50 dilution of hybridoma supernatant) recognizing DNA damage produced by the photoactivated drug 8-methoxypsoralen [15] or with antibody pre-absorbed with MDA-DNA (4.5 µg/ml for 30 min at 37°C) before use. MDA-DNA used for pre-absorption was prepared as described previously [16]. A Cell Analysis microscope was used to measure the relative intensity of nuclear staining in 50 randomly selected cells. Data presented are the object average optical density multiplied by 1000.

Statistic analysis

The difference in mean value of relative staining intensity between smokers and non-smokers was analyzed by paired *t*-test. Two sided *P* values were calculated and values <0.05 were considered significant. Data were also analyzed after being log transformed to normalize the distribution.

Results

Specific nuclear staining was observed in cells treated with MDA but not in untreated cells with representative staining as illustrated in Figure 1A and B, respectively. Quantification of staining indicated a dose-related increase in staining with relative staining intensities of 29 ± 5 , 46 ± 4 , 109 ± 22 , 115 ± 24 in cells treated with 0, 10, 20 and 40 mM MDA, respectively. The immunoperoxidase method was then

applied to the detection of damage in oral mucosa cells from smokers and non-smokers. The coefficient of variation for the positive and negative controls was 12 and 10%, respectively ($n = 4$). One-way analysis of variance indicated there was no significant difference in repeat staining of WC3 cells. Representative staining in a smoker (No. 44) and non-smoker (No. 34) are illustrated in Figure 1C and D, respectively. As additional controls for staining specificity, oral cells of a smoker not paired to a non-smoker were stained. Pre-absorption of the primary antibody with MDA-DNA before use decreased staining from 109 ± 39 to 63 ± 9 (Figure 1E). Pre-treatment with DNase decreased relative staining to 43 ± 7 (Figure 1F) whereas staining with a non-specific antibody recognizing DNA damage produced by 8-methoxypsoralen gave a value of 45 ± 9 for the same subject.

Quantification of staining intensity for all subjects is given in Table I. Higher levels of specific nuclear staining were observed in most smokers compared with their matched non-smokers. The distribution of staining in smokers and non-smokers is given in Figure 2. Variation in staining was ~2.5-fold in non-smokers (range from 47 ± 15 to 117 ± 46) and 4.5-fold in smokers (from 44 ± 7 to 197 ± 66). The mean level of relative staining intensity was elevated 1.3-fold in smokers (mean 97 ± 41) compared with non-smokers (mean 74 ± 17 , $P = 0.02$). After log transformation the mean value in smokers was 4.49 ± 0.42 and in non-smokers 4.29 ± 0.23 ($P = 0.04$).

Discussion

DNA damage in humans is the result of exposure to exogenous chemicals, including environmental pollutants or their metabolites as well as normal metabolic processes. Several studies have emphasized the importance of the identification, characterization and quantitative determination of electrophiles of endogenous origin [17–19]. It is now known that significant DNA damage occurs

endogenously through interaction with reactive chemical species, which arise during normal metabolism or through endogenous processes such as oxidation, methylation, deamination, depurination [20–23] and inflammation [18]. Thus, endogenous DNA damage may contribute to the aetiology of human genetic disease and cancer.

Lipid peroxidation generates a complex variety of products many of which are reactive electrophiles; MDA appears to be the most mutagenic and carcinogenic [3]. In the current study, an immunoperoxidase method was developed to detect MDA–DNA adducts using a previously developed monoclonal antibody and it was applied to the detection of endogenous damage in human oral mucosa cells. A relatively large interindividual variation in adduct levels was observed, \approx 2.5-fold in non-smokers and 4.5-fold in smokers (Table I). Similar or even higher variation was reported in previous human studies for other types of DNA damage [6,9]. This is most likely a result of genetically determined interindividual differences in the rate and extent of intracellular oxidation processes and/or differences in DNA repair rates [24]. Cigarette smoke, in both the gaseous phase and condensed particles, contains alkenes, nitrosamines, aromatic and heterocyclic hydrocarbons and amines. In addition, it is an excellent source of reactive oxygen species (ROS), such as hydroxyl radicals, superoxides and peroxides that are capable of initiating or promoting oxidative damage [25–27]. Reaction of the ROS with cellular DNA results in oxidative damage, including a number of oxidized bases, single-strand breaks and/or alkali-labile lesion [28]. These types of DNA damage are considered to be crucial in cancer development, thus providing an additional possible mechanism for the apparent association between smoking and mouth, lung, pharynx, esophagus, bladder and cervical cancers [28–30]. A 1.3-fold higher level of specific nuclear staining for MDA–DNA in smokers compared with that in

non-smokers was observed. This is somewhat lower than the 1.7–2-fold differences observed when staining oral cells of smokers and non-smokers for the bulky PAH- and 4-ABP–DNA adducts or 8-hydroxy or oxo-deoxyguanosine, a useful marker of DNA damage produced by oxygen radicals [14]. Diet, alcohol consumption, carcinogen exposure, certain diseases and genetic factors have been associated with the extent of lipid peroxidation [12]. These factors, which were unmeasured in the present study, may account for the smaller differences observed in MDA–DNA adducts compared with those of the bulky carcinogens.

Like other analytic methods, the immunohistochemical assay has advantages and disadvantages. The major advantages are its ability to detect DNA damage in specific cell types within a tissue, and its applicability to small amounts of sample, even single cells. If used on blood cells, <1 ml of blood is required and DNA isolation is not necessary. Staining can be evaluated in a qualitative or semiquantitative manner. Qualitative assessment uses a subjective estimation of staining intensity and/or number of positively stained cells with an arbitrary scale whereas semiquantitative methods measure level of staining in the cells. The major disadvantage of the method is that absolute quantification, in terms of adducts per nucleotide, is not possible and data are expressed as relative staining intensity. This is not a problem for most studies in which subjects with different exposures or disease states are compared. In addition, the immunohistochemistry method is generally not as sensitive as competitive ELISA, slot blot, GC/MS or post-labeling. Although the MDA–DNA antibody appears to be highly specific for this adduct [1], antibody cross-reactivity with other adducts with a similar structure may potentially cause problems in quantification.

Conclusion

It is concluded that the immunoperoxidase method is sufficiently sensitive to be useful

in the detection of MDA–DNA adducts in human samples. MDA–DNA adducts may serve a biomarker of DNA damage by lipid

peroxidation induced endogenously or exogenously.

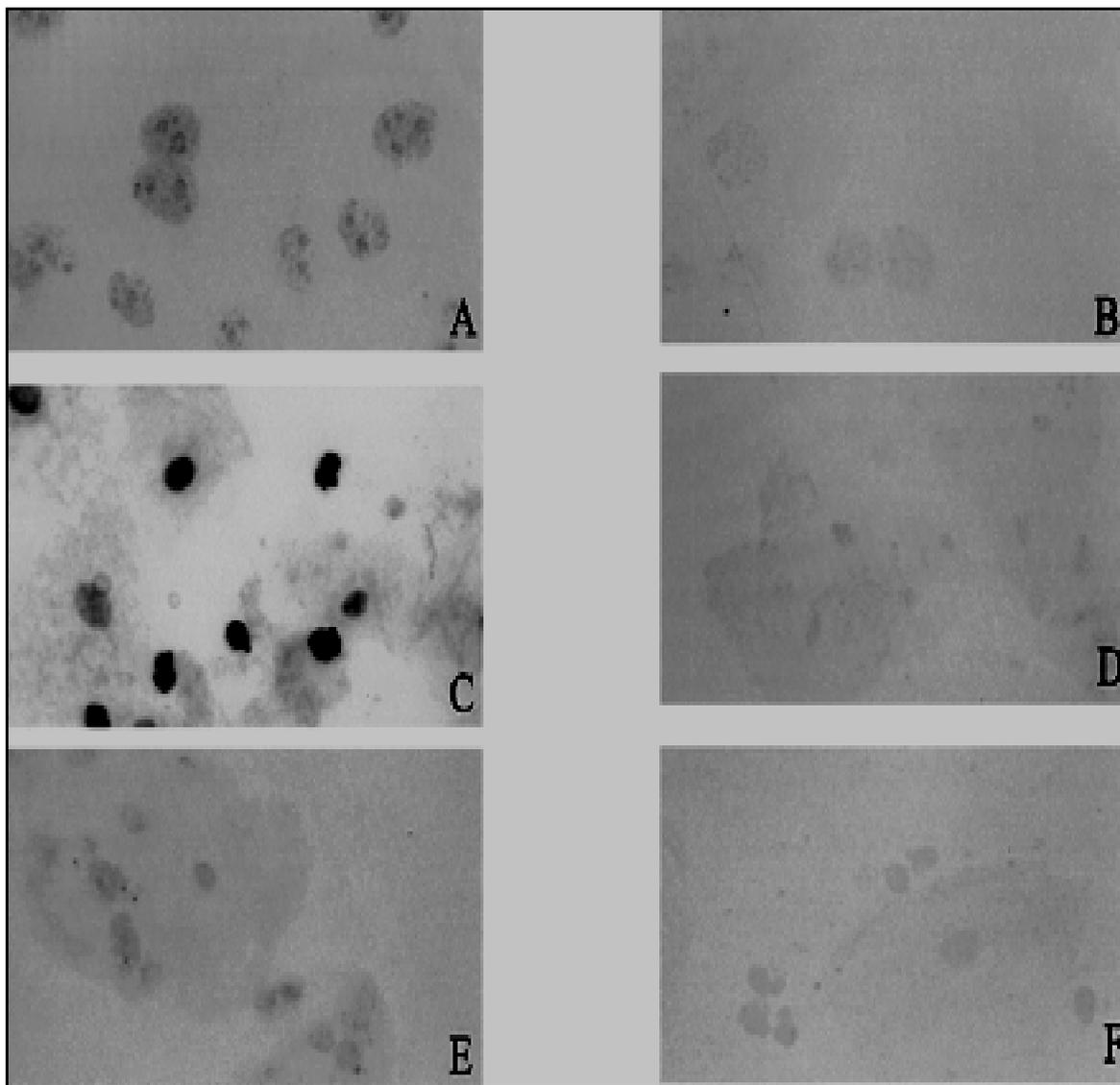


Fig. 1. Immunohistochemical staining of liver cells in mice and human oral mucosa cells with monoclonal antibody D10A1 recognizing MDA–DNA adducts. Staining of liver cells treated with 20 mM MDA (A) or untreated (B); oral cells from a smoker (C) and non-smoker (D); oral cells of a smoker stained with antibody that was pre-absorbed with MDA–DNA before use (E); and oral cells of a smoker pre-treated with DNase before staining (F) (x400).

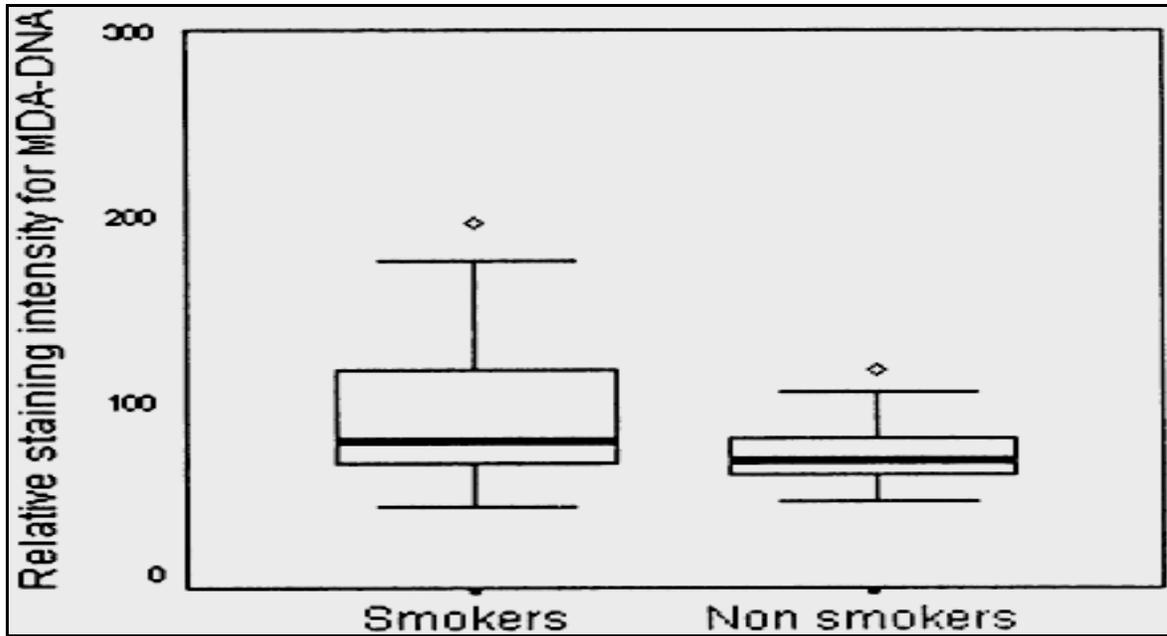


Fig. 2. Comparison of relative intensity of staining of oral mucosa cells of smokers and non-smokers for MDA–DNA damage. The lower and upper edges of the box are the 25 and 75 percentile values. Median values are shown by the lines within the boxes.

Table I. Immunoperoxidase staining of oral mucosa cells from smokers and non-smokers for MDA–DNA damage

Non-smokers				Smokers			
Sex ^b	ID ^c	Age	Mean ± SD ^d	ID	Age	Cigarette/day	Mean ± SD
M	32	47	47 ± 15	1	42	20	72 ± 19
F	51	26	69 ± 25	2	27	20	120 ± 32
F	36	49	75 ± 67	3	47	20	78 ± 30
M	38	28	58 ± 9	4	32	40	77 ± 28
M	47	52	67 ± 19	5	51	20	173 ± 45
F	52	54	90 ± 23	6	50	20	176 ± 50
F	53	47	78 ± 14	7	46	30	150 ± 49
F	34	40	64 ± 2	8	40	40	117 ± 39
M	37	45	55 ± 16	9	42	40	98 ± 33
F	41	30	80 ± 19	10	30	25	108 ± 37
M	40	25	54 ± 19	11	30	20	69 ± 46
M	45	36	61 ± 19	12	34	20	78 ± 20
F	39	30	58 ± 21	13	25	24	72 ± 22
F	43	26	63 ± 42	14	28	30	59 ± 32
M	35	50	117 ± 56	15	47	20	106 ± 32
M	46	29	70 ± 43	16	29	20	96 ± 53
F	31	36	66 ± 19	17	36	22	77 ± 28

M	44	50	99 ± 33	18	49	25	197 ± 66
M	42	36	69 ± 15	19	33	20	44 ± 7
F	33	38	62 ± 21	20	36	35	49 ± 14
F	49	39	80 ± 33	21	34	20	53 ± 12
F	48	51	81 ± 23	22	48	30	53 ± 20
F	50	46	107 ± 43	23	46	40	68 ± 13
F	54	49	74 ± 51	24	43	22	150 ± 69
M	55	53	106 ± 34	25	50	30	86 ± 48
^b M, male; F, female.							
^c ID, subject number.							
^d Relative staining intensity measured in 50 cells/subject.							

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