Formaldehyde promotes and inhibits the proliferation of cultured tumour and endothelial cells

E. Tyihák*, J. Bocsi†, F. Timár†, G. Rácz† and B. Szende†

*Plant Protection Institute, Hungarian Academy of Sciences and †1st Institute of Pathology and Experimental Cancer Research, Molecular Pathology Research Group of the Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary

Received 7 June 2000; revision accepted 6 December 2000

Abstract. Formaldehyde was applied in various doses (0.1–10.0 mM) to HT-29 human colon carcinoma and HUV-EC-C human endothelial cell cultures. Cell number, apoptotic and mitotic index as well as proportion of cells in S-phase was investigated by morphological methods and flow cytometry. Ten mM of formaldehyde caused high degree of cell damage and practically eradicated the cell cultures. One mM of formaldehyde enhanced apoptosis and reduced mitosis in both types of cell cultures, in a moderate manner. The low dose (0.1 mM) enhanced cell proliferation and decreased apoptotic activity of the cultured cells, the tumour cells appeared to be more sensitive. The possible role of this dose-dependent effect of formaldehyde in various pathological conditions, such as carcinogenesis and atherogenesis is discussed with emphasis on the eventual interaction between formaldehyde and hydrogen peroxide.

INTRODUCTION

It has recently been demonstrated that formaldehyde (HCHO) – similar to hydrogen peroxide (H₂O₂), another small molecule (Ahmad 1995; Lord-Fontaine 1999) – is an endogenous component, mainly in the form of hydroxymethyl groups, of all biological systems (Tyihák *et al.* 1998a,b). It can be formed during enzymatic methylation and demethylation processes (Husztai & Tyihák 1986; Fannin & Bush 1992).

S-Adenosyl-L-methionine (SAM) serves as a methyl donor in a vast number of enzymatic transmethylation reactions including DNA methylation (Brandeis *et al.* 1993). The formation of HCHO from the S-methyl group of SAM has been demonstrated in enzymatic transmethylation reaction (Husztai & Tyihák 1986). This observation raises the question of the existence of a methyl cation or methyl radical in this enzymatic process, that is, whether the enzymatic formation of methyl groups takes place through HCHO (Tyihák *et al.* 1998b). HCHO is also formed within the cell by oxidative demethylation (via demethylases and/or special peroxidases) of a variety of endogenous and/or exogenous N-, O- and S-methyl compounds. During this demethylation process HCHO and the demethylated compound can be formed (Kawata *et al.* 1983; Kedderis & Hollenberg 1983; Kucharczyk *et al.* 1984).

It became increasingly evident that there is a primary HCHO cycle in biological systems in which formation of L-methionine from L-homocysteine and the HCHO-yielding function of

SAM are essential components of such a fundamental biological pathway system (Tyihák *et al.* 1998a,b). The abnormalities of the originally controlled HCHO cycle (Tyihák *et al.* 1998a,b) and the uncontrolled enzymatic production of HCHO, e.g. by semicarbazide-sensitive amino oxydase (SSAO) from endogenous and/or exogenous substrates (e.g. nicotine, adrenaline) – among others – through methylamine (Yu 1990; Boor *et al.* 1992; Yu *et al.* 1997a; Garpenstrand *et al.* 1999), may be potential risk factors in the pathogenesis of various disorders.

It is well-known that the aqueous solution of HCHO (formalin solution) has a concentration-dependent cell proliferation inhibiting activity, that is, it has an antibacterial and antimitotic effect in general (Walker 1964; Swenberg *et al.* 1983). It has also been reported that exogenous HCHO could be both genotoxic and carcinogenic (Monticello & Morgan 1997; Merk & Speit 1998). The possible role of HCHO in the apoptotic process was also proposed (Szendé *et al.* 1995; Szendé *et al.* 1998).

It seems from these recent observations that HCHO as simplest aliphatic aldehyde may be a key molecule in biological systems particularly in cell proliferation. The present paper summarizes our preliminary results gained on the effects of diluted formalin solution on the proliferation and apoptosis of cultured tumour cells and endothelial cells based on experience with various levels of HCHO in animal tissues (Tyihák *et al.* 1998b). Since the role of formaldehyde in the induction of neoplasia and atherosclerosis has been widely discussed (Conolly *et al.* 1995; Monticello & Morgan 1997; Yu *et al.* 1997b; 1998), it seemed to be advisable to use both tumour and endothelial cells in our experiments.

MATERIALS AND METHODS

Cell cultures

The human colon carcinoma cell lines HT-29 (ATCC HTB-38) and the human endothelial cell line HUV-EC-C (ATCC CRL-1730) were used. The monolayer cell cultures were maintained in 24-well and six-well Greiner plates (Kremsmünster, Austria) using RPMI supplemented with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO). The number of cells at plating was 10^4 /ml. For morphological studies, cover glasses were put on the bottom of wells in six-well plates. At termination, the cells on the cover glasses were fixed with methanol and stained with haematoxylin and eosin (HE).

Treatment of the cell cultures

The colon carcinoma cells and the endothelial cells were treated at hour 48 after plating. Samples for evaluation were taken 24, 48 and 72 h after treatment. The treatment was performed with 0.1, 1.0, 10.0 mM formaldehyde in triplicate samples. Formaldehyde (analytical purity) was obtained from Lachema n.p. (Brno, Czech Republic).

Evaluation

The cells were counted in Buerker chambre, determining mitotic and apoptotic rate in stained samples and by flow cytometry. Both attached and floating cells were considered. For determination of apoptosis index attached cells were fixed and stained on the cover glass itself, whereas floating cells were fixed and stained after centrifugation of the culture medium at $40g \cdot 5'$ using a cytocentrifuge (Cytospin, Shandon). Similarly, floating cells in the culture medium and cells detached from the bottom of the wells by mild trypsinization (0.25%) were used for flow cytometry. Apoptotic cells were recognized using criteria of Wyllie *et al.* (1986). The apoptotic and mitotic index was determined by counting 2000 cells and expressed in per centage.

Flow cytometry

For this purpose 2×10^5 /ml HT-29 cells were plated in 24-well plates. The dose of formaldehyde was 0.1, 0.5 and 1.0 mM. At termination of the experiment cells were collected and fixed 70% ethanol at -20°C for 24 h. Flow cytometry (DNA content measurement) of HT-29 cells was performed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) in conjugation with Macintosh Quadra computer and Cellquest data acquisition package. Estimation of the proportion of apoptotic cells and the cell cycle analysis were performed using Modfit software. Cell suspension samples were prepared as described by Gong *et al.* (1994), Mihalik *et al.* (1995), Schuler *et al.* (1994). Briefly, after ethanol fixation the internucleosomally fragmented DNA was removed from apoptotic cells in citrate-phosphate buffer (pH = 7.8) supplemented with Rnase, afterwards the DNA content was determined by flow cytometry. The control and four samples were measured for each dose.

Statistical analysis

ANOVA test was applied for $\pm$ SD and significance.

RESULTS

Cell number

Tables 1 and 2 show the number of HT-29 and HUV-EC-C cells, untreated and treated with various doses of formaldehyde. Ten mM of formaldehyde resulted in total cell kill in both types of cultured cells. With 1.0 mM formaldehyde marked and significant decrease of cell number in

Table 1 Effect of various doses of formaldehyde on cell number of HT-29 human colon carcinoma cell culture

Treatment	Cell number ($\times 104$) on subsequent days after treatment		
	Day 1	Day 2	Day 3
Control	3.96 ± 0.9	4.3 ± 0.3	3.66 ± 0.57
10.0 mM	—	—	—
1.0 mM	$1.3 \pm 0^*$	$1.06 \pm 0.2^\dagger$	$0.88 \pm 0.13^*$
0.1 mM	3.86 ± 0.23	$9.3 \pm 0^\dagger$	$11.0 \pm 1.0^\dagger$

*Values are average of three samples $\pm$ SD $P < 0.01$; † Values are average of three samples $\pm$ SD $P < 0.0001$.

Table 2 Effect of various doses of formaldehyde on cell number of HUV-EC-C endothelial cell culture

Treatment	Cell number ($\times 104$) on subsequent days after treatment		
	Day 1	Day 2	Day 3
Control	5.63 ± 0.32	7.9 ± 0.65	10.33 ± 0.58
10.0 mM	—	—	—
1.0 mM	4.4 ± 0.36	6.5 ± 0.3	8.7 ± 0.26
0.1 mM	$7.43 \pm 0.5^*$	$12.46 \pm 0.45^\dagger$	$17.56 \pm 0.6^\dagger$

*Values are average of three samples $\pm$ SD $P < 0.01$; † Values are average of three samples $\pm$ SD $P < 0.0001$.

Table 3 Effect of various doses of formaldehyde on apoptotic (A) and mitotic (M) index of HT-29 human colon carcinoma cell culture

Treatment	Apoptotic (A) and mitotic (M) index (%) on subsequent days after treatment					
	Day 1		Day 2		Day 3	
	A	M	A	M	A	M
Control	2.66 ± 0.57	3.0 ± 0	3.33 ± 0.57	2.66 ± 0.57	3.66 ± 0.57	2.33 ± 0.57
10.0 mM	—	—	—	—	—	—
1.0 mM	4.66 ± 0.57*	0.66 ± 0.57	5.33 ± 0.57*	0.66 ± 0.57*	4.33 ± 0.57	1.0 ± 0
0.1 mM	2.33 ± 0.57	3.33 ± 0.57	1.33 ± 0.57*	5.66 ± .57†	1.0 ± 0	6.33 ± 0.57‡

*Values are average of three samples ± SD $P < 0.01$; †Values are average of three samples ± SD $P < 0.003$;

‡Values are average of three samples ± SD $P < 0.001$.

Table 4 Effect of various doses of formaldehyde on apoptotic (A) and mitotic (M) index of HUV-EC-C endothelial cell culture

Treatment	Apoptotic (A) and mitotic (M) index (%) on subsequent days after treatment					
	Day 1		Day 2		Day 3	
	A	M	A	M	A	M
Control	1.66 ± 0.57	3.66 ± 0.57	2.0 ± 0	4.33 ± 0.57	2.33 ± 0.57	2.66 ± 0.57
10.0 mM	—	—	—	—	—	—
1.0 mM	1.66 ± 0.57	3.0 ± 0.57	2.66 ± 0.57	3.33 ± 0.57	3.33 ± 0.57	2.0 ± 0
0.1 mM	0.66 ± 0.57	4.33 ± 0.57	1.0 ± 0	5.0 ± 1.0	1.0 ± 0	4.33 ± 0.57*

*Values are average of three samples ± SD $P < 0.02$.

HT-29 cultures was seen. Less significant decrease in cell number was observed after treatment of HUV-EC-C cells with the same dose of formaldehyde.

On the other hand, the cell numbers of both cell lines significantly increased 48 and 72 h after treatment with 0.1 mM formaldehyde. This effect was even more significant in case of colon carcinoma cells, compared to the endothelial cells.

Mitotic and apoptotic index

As summarized in Tables 3 and 4, no mitotic or apoptotic figures could be recognized in the cultures treated with 10.0 mM formaldehyde, due to nearly total cell loss and destruction of the structure of the remaining few cells. In both cell lines, 1.0 mM formaldehyde slightly increased the apoptotic index and decreased the mitotic index.

The effect of 0.1 mM formaldehyde was characterized 48 and 72 h after treatment by increased mitotic and decreased apoptotic activity compared to control in both cell lines.

Flow cytometry

The apoptotic ratio observed by flow cytometry was in good correlation with that counted on the base of morphology. Both 0.1 and 0.5 mM formaldehyde decreased the apoptotic ratio. In

Table 5 Apoptotic fraction and s phase ratio in untreated and formaldehyde-treated cell cultures. Measured by flow cytometry

Treatment	Apoptosis (%)	G ₀ /1 (%)	S phase (%)	G ₂ + M
Control	3.5 ± 0.2	70.0 ± 1.1	19.5 ± 2.3	11.2 ± 1.0
0.1 mM formaldehyde	2.6 ± 0.4†	70.1 ± 3.1	19.1 ± 3.2	11.3 ± 2.9
0.5 mM formaldehyde	2.9 ± 0.2*	66.7 ± 2.4*	27.8 ± 1.7‡	5.2 ± 1.0‡

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

higher dose (1.0 mM) the flow cytometric method was inappropriate because of fixation of fragmented DNA, caused by formaldehyde. In the 0.5 mM formaldehyde treated cultures the proportion of cells in S-phase increased significantly compared to the control (Table 5).

DISCUSSION

Our previous studies showed that the pro-apoptotic activity of hydroxymethylated arginines and 1'-methyl-ascorbigen can be generated from the HCHO formed from their N-hydroxymethyl or N-methyl groups (Szende *et al.* 1995; Szende *et al.* 1998).

In our present study, apoptosis of not only tumour cells (Grafstrom 1998), but also endothelial cells could be induced by 1.0 mM HCHO, although this effect was relatively moderate when compared with that exerted on the tumour cell culture. The most important finding, however, was that a low dose of HCHO (0.1 mM) inhibited apoptotic and enhanced proliferative activity in both types of cultured cells, the tumour cells being again more responsive than the endothelial cells. It has to be pointed out that the doses of HCHO were designed considering the concentrations of this compound likely to appear in the cells or on the cell surface in form of labile hydroxymethyl groups as an aftermath of intra- and extracellular biochemical reactions (Tyihák *et al.* 1998b).

HCHO has been claimed to be involved as a normal and indispensable component in all cells (Tyihák *et al.* 1998a,b) as well as a pathogenic factor in various pathological processes such as carcinogenesis (Swenberg *et al.* 1983; Conolly *et al.* 1995; Shaham *et al.* 1996) and atherogenesis (Boor *et al.* 1992; Yu *et al.* 1997a). In carcinogenesis antiapoptotic and in atherogenesis pro-apoptotic activity had postulated. This contradiction may be at least partially interpreted regarding the dose-dependent antiapoptotic or pro-apoptotic (pro-proliferative or antiproliferative) effect of HCHO. Relatively low doses of exogenous or intrinsic HCHO may enhance cell proliferation and inhibit apoptosis, leading even to neoplasia. Higher doses, on the other hand, may cause damage to endothelial, epithelial or other cells by inducing apoptosis and inhibiting repair by cell proliferation.

It is known that HCHO and H₂O₂ as small molecules can continuously be formed intra- and extracellularly (Kucharczyk *et al.* 1984; Heck *et al.* 1990; Tyihák *et al.* 1998a,b). H₂O₂ is a stable nonradical molecule which is produced endogenously by divalent reduction of molecular oxygen via miscellaneous oxidases or by dismutation of the superoxide anion (Fridovich 1995) and at that time the HCHO is a very reactive aliphatic aldehyde therefore it occurs mainly in the cells in the form of labile hydroxymethyl groups (Heck *et al.* 1990; Tyihák *et al.* 1998a,b). It is possible that HCHO and H₂O₂ can meet in different regions of cells and they can interact

endogenously. In this interaction the very reactive single oxygen and excited HCHO can be formed (Trézl & Pipek 1988; Tyihák *et al.* 1994). The invasive (stress situation) or noninvasive (stress-free condition) formation of these excited molecules plays a role in disease resistance killing the pathogens or tumour cells, however, they also could be potential risk factors of various disorders (Tyihák *et al.* 1994). The potential risk for vascular disorders of the interaction between HCHO and H₂O₂ is excellently illustrated by metabolism of nicotine (Yu 1998). Methylamine as the end product of nicotine metabolism by semicarbazide-sensitive amine oxydase (SSAO) is readily deaminated *in vitro* and *in vivo* (Yu 1997b) and HCHO and ammonia can be formed as well as hydrogen peroxide is generated from water simultaneously (Heck *et al.* 1990). Similar deamination reaction is demonstrated for adrenaline (Yu *et al.* 1997a), sarcosine, creatinine (Dar *et al.* 1985).

It is especially interesting that exogenous application of H₂O₂ can induce apoptosis, however, there is always a level of HCHO in the given cell systems so there is a possibility for interaction between these two small molecules. Similar situation occurs if we apply HCHO to cells. In this case exogenous HCHO may meet endogenous H₂O₂. These two small molecules can not be avoided in cell proliferation and apoptosis, however, further investigations are needed to clarify the real responsible molecule in these processes.

REFERENCES

- AHMAD S (1995) *Oxidative Stress and Antioxidant Defenses in Biology*. Chapman & Hall, New York.
- BOOR PJ, TRENT MB, LYLES GA, TAO M, ANSARI GAS (1992) Methylamine metabolism to formaldehyde by vascular semicarbazide-sensitive amino oxydase. *Toxicology* **73**, 251–258.
- BRANDEIS M, ARIEL M, CEDAR H (1993) Dynamics of DNA methylation during development. *Bioassays* **15**, 709–713.
- CONOLLY RB, ANDJELKOVICH DA, CASANOVA M, HECK HD, JANSZEN DB, KIMBELL JS, MORGAN KT, RECIO L (1995) Multidisciplinary, iterative examination of the mechanism of formaldehyde carcinogenicity: the basis for better risk assessment. *CIIT Activities* **15**, 1–16.
- DAR MS, MORSELLI PL, BOWMAN ER (1985) The enzymatic systems involved in the mammalian metabolism of methylamine. *Gen. Pharmacol.* **16**, 557–560.
- FANNIN FF, BUSH LP (1992) Nicotine demethylation in nicotiana. *Med. Sci. Res.* **20**, 867–868.
- FRIDOVICH I (1995) Superoxide radical and superoxide dismutases. *Ann. Rev. Biochem.* **64**, 97–112.
- GARPENSTRAND H, EKBLOM J, VON ARBIN M, ORELAND L, MURRAY V (1999) Plasma semicarbazide-sensitive amino oxydase in stroke. *Eur. Neurol.* **41**, 20–23.
- GONG J, TRAGANOS F, DARZYNKIEWICZ Z (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.* **218**, 314–319.
- GRASFSTROM RC (1998) Mechanisms of formaldehyde toxicity in cultured human cells. 4th Intern. Conference On the Role of Formaldehyde in Biological Systems, July 1–4, 1998, Budapest, Hungary, (Abstract) Book, p. 13.
- HECK H, CASANOVA M, STARR TB (1990) Formaldehyde toxicity – new understanding. *Crit. Rev. Toxicol.* **20**, 397–426.
- HUSZTI S, TYIHÁK E (1986) Formation of formaldehyde from S-adenosyl-L-(methyl-3H) methionine during enzymic transmethylation of histamine. *FEBS Lett* **209**, 362–366.
- KAWATA S, SUGIYAMA T, IAMI J, MINAMI Y, TARUI S, OKAMOTO M, YAMANO T (1983) Hepatic microsomal cytochrome P-450 dependent N-demethylation of methylguanidine. *Biochem. Pharmacol.* **32**, 3723–3728.
- KEDDERIS GL, HOLLENBERG PF (1983) Peroxidase-catalysed N-demethylation reactions. *J. Biol. Chem.* **259**, 663–668.
- KUCHARCZYK N, YANG JT, WONG KK, SOFIA RD (1984) The formaldehyde-donating activity of N5, N10-methylene tetrahydrofolic acid in xenobiotic biotransformation. *Xenobiotica* **14**, 667–676.
- LORD-FONTAINE ST, AVERILL DA (1999) Enhancement of cytotoxicity of hydrogen peroxide by hyperthermia in Chinese hamster ovary cells: role of antioxidant defenses. *Archiv. Biochem. Biophys.* **363**, 283–295.
- MERK O, SPEIT G (1998) Significance of formaldehyde-induced DNA-protein crosslinks for mutagenesis. *Environ. Mol. Mutagen.* **32**, 260–268.
- MIHALIK R, KOPPER L, BENICZUR M (1995) Modulation of drug-induced apoptosis in a human B-lymphoma cell line (HT58). *Immunol. Letter* **48**, 17–21.

- MONTICELLO TM, MORGAN KT (1997) Chemically-induced nasal carcinogenesis and epithelial cell proliferation: a brief review. *Mutat. Res.* **380**, 33–41.
- SCHULER D, SZENDE B, BORSI JD, MARTON T, BOCSI J, MAGYAROSI E, KOÓS R, CSÓKA ML (1994) Apoptosis as a possible way of destruction of lymphoblasts, after glucocorticoid treatment of children with acute lymphoblastic leukemia. *Pediatric Hematol. Oncol.* **11**, 641–649.
- SHAHAM J, BOMSTEIN Z, MELTZER A, KAUFMAN Z, PALMA E, RIBAK J (1996) DNA-protein crosslinks, a biomarker of exposure to formaldehyde – in vitro and in vivo studies. *Carcinogenesis* **17**, 121–125.
- SWENBERG JA, GROSS EA, RANDALL HW, BARROW CS (1983) The effect of formaldehyde exposure on cytotoxicity and cell proliferation in formaldehyde. In: Clary JJ, Gibson JE, Waritz RS, eds. *Toxicology, Epidemiology, Mechanisms*. Marcel Dekker, New York.
- SZENDE B, TYIHÁK E, SZÓKÁN GY, KÁTAY GY (1995) Possible role of formaldehyde in the apoptotic and mitotic effect of 1'-methyl-ascorbigen. *Path. Oncol. Res.* **1**, 38–42.
- SZENDE B, TYIHÁK E, TRÉZL L, SZÓKE É, LÁSZLÓ I, KÁTAY GY, KIRÁLY-VÉGHÉLY ZS (1998) Formaldehyde generators and capturers as influencing factors of mitotic and apoptotic processes. *Acta Biol. Hung.* **49**, 323–329.
- TRÉZL L, PIPEK J (1988) Formation of excited formaldehyde in model reactions simulating real biological systems. *J. Mol. Struct. (Theochem.)* **170**, 213–223.
- TYIHÁK E, ALBERT L, NÉMETH ZSI, KÁTAY GY, KIRÁLY-VÉGHÉLY ZS, SZENDE B (1998B) Formaldehyde cycle and the natural formaldehyde generators and capturers. *Acta Biol. Hung.* **49**, 225–238.
- TYIHÁK E, ROZSNYAY S, SÁRDI E, GULLNER G, TRÉZL L, GÁBORJÁNYI R (1994) Possibility of formation of excited formaldehyde and singlet oxygen in biotic and abiotic stress situations. *Acta Biol. Hung.* **45**, 3–10.
- TYIHÁK E, TRÉZL L, SZENDE B (1998a) Formaldehyde cycle and the phases of stress syndrome. *Ann. N. Y. Acad. Sci.* **851**, 259–270.
- WALKER JF (1964) Formaldehyde. Krieger, New York.
- WYLLIE AH, KERR JFR, CURRIE AR (1986) Cell death: the significance of apoptosis. *In Rev. Cytol.* **68**, 251–306.
- YU P (1990) Oxidative deamination of aliphatic amines by rat aorta semicarbazide – sensitive amine oxidase. *J. Pharm. Pharmacol.* **2**, 61–66.
- YU PH (1997b) Deamination of methylamine and angiopathy; toxicity of formaldehyde, oxidative stress and relevance to protein glycoxidation in diabetes. *J. Neural. Transm.* **52**, 207–222.
- YU PH (1998) Increase of formation of methylamine and formaldehyde in vivo after administration of nicotine and the potential cytotoxicity. *Neurochem. Res.* **23**, 1205–1210.
- YU PH, LAI CT, ZUO DM (1997a) Formation of formaldehyde from adrenaline: a potential risk factor for stress-related angiopathy. *Neurochem. Res.* **22**, 615–620.