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ARTICLE

Possible Role of Formaldehyde in the Apoptotic and Mitotic Effect of 1-Methyl-Ascorbigen

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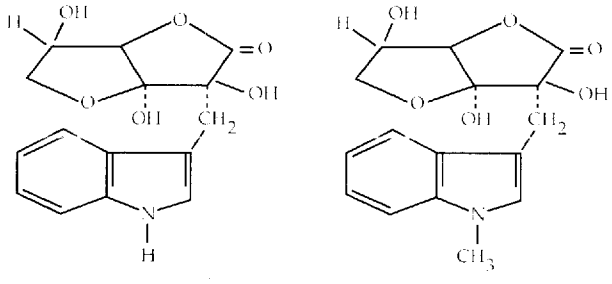
PC-3 human prostate carcinoma cells were treated with 100 µg/ml 1-methyl-ascorbigen (Me-Asc). This treatment resulted in a significant decrease in tumor cell number in parallel with an increase in apoptotic cells. The formaldehyde (HCHO) level in the culture medium was also increased. Dimedone (Di), a known capture molecule forming formaldemethone with HCHO, applied simultaneously with Me-Asc in 10 µg/ml doses diminished the apoptosis-inducing effect of Me-Asc. The possible role of in situ generated HCHO in the induction of apoptosis is discussed. (Pathology Oncology Research Vol 1, No1, 38–42, 1995)

Key words: formaldehyde, apoptosis, methylascorbigen

Introduction

Ascorbigen (Asc), an indole-containing derivative of L-ascorbic acid (AA), can be isolated from some fresh and cooked cruciferous-vegetable tissues.[1,2] This bound AA is a major product of alkaloid glucobrassicin biotransformation and one of the important factors responsible for the anticarcinogenic properties of a cruciferous-vegetable diet.[3] The synthesis of Asc and its analogs was carried out for systematic pharmacological investigations.[4-6] Their biological evaluation showed that some of the synthesized analogs are immunomodulators.[7] The most active is 1-methyl-ascorbigen (Me-Asc) *(Fig.1.)*, which has immunomodulating activity (increases activity of macrophages, neutrophils, T-killers and NK cells and enhances production of tumor necrosis factor)[8] and exerts therapeutic effects (inhibition of tumor growth, protection of animals from bacterial and viral infections). It was suggested that the immunomodulating activity of 1-Me-Asc may also be responsible for its antitumoral effects.[8] It is of importance that among the derivatives of Asc, Me-Asc has high biological activity. All N-methyl group containing substances are potential HCHO generators, including ethylmorphine,[9] epinephrine[10] and nicotine[11] and in situ generated HCHO may play a role in the diverse biological activities of these compounds through as yet unknown mechanisms. Previously we have demonstrated that the endogenously found amino acid N-trimethyl-L-lysine (TML) has an immunostimulating and cell proliferation promoting effect in animal and human test systems and that methyl groups play a role in this activity.[12,13] Accordingly, the N-methyl group was hypothesized to play an essential role in the diverse biological activities of Me-Asc.

The aim of the present paper is to demonstrate the *in vitro* effects of Me-Asc on tumor cells, with special emphases on its apoptotic and mitotic activities. The role of HCHO in the mode of action of Me-Asc was also investigated.



Ascorbigen 1'-methyl-ascorbigen

Figure 1. *The chemical structure of ascorbigen and 1-methyl ascorbigen.*

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Materials and methods

Chemical procedures

Synthesis of Me-Asc was carried essentially according to the previously described methods.[4,6,11]

Determination of HCHO in the culture medium

The cellfree culture medium was mixed 1:1 with dimedone solution (0.01% dimedone in 5% acetic acid solution). The formaldemethone (a dimedone adduct of HCHO) formed was extracted with chloroform, then filtered through sicc. Na_2SO_4. The clear water-free solution was used in overpressured layer chromatographic (OPLC) separation[15-18] of formaldemethone, carried out by OPLC in a CHROMPRES 25 OPLC instrument (Laboratory Instrument Co., Ltd., Budapest, Hungary) using a chloroform-methylene chloride eluent mixture (35:65, v/v) and HPTLC silica gel 60 F_{254} chromatoplate (Merck Co., Darmstadt, Germany) with impregnated edges.[19] The plate was dried after the separation in a cold stream of air for 2 min. Quantification was conducted in the reflectance mode at 265 nm using a Shimadzu CS 930 scanner (Shimadzu Co., Kyoto, Japan) and a calibration curve.

Biological studies

PC-3 human prostate carcinoma cells were plated in 6-well Greiner dishes (Nürtingen, Germany). The culture medium was RPMI supplemented with 10% Calf serum (Flow, Irwine, Scotland). The dishes were placed into a humidified 5% CO_2, 37°C atmosphere. Cells were plated at 10^4cells/well. Triplicates were used for each treatment group and time point. Treatments started 24 hours after plating. 1-Me-Asc (100 µg/ml) and Di (10 µg/ml) were dissolved in RPMI. No serum was used in the culture medium after treatment and the medium was not changed during the subsequent 72 hours. The treatment schedule was as follows: 1. Control; 2. Me-Asc: 100 µg/ml; 3. Di:10 µg/ml; 4. Me-Asc 100 µg/ml + Di: 10 µg/ml. The doses were chosen on the basis of our previous experiments.[20] Cell counts were taken and the cell viability was determined by methylen blue exclusion 24, 48 and 72 hours after treatment. For morphological studies cover slips were placed into 5 ml Greiner flasks and 10^4 PC-3 cells/ml were grown on their surface. Five flasks per treatment group were used. The treatment schedule was the same as described above. All cultures were fixed after 48 hours of treatment in 3:1 of methanol: 10% acetic acid. Three cover-slips from each treatment group were stained with HE and two of each group were exposed to immunocytochemical reagents using Apop-Tag (Oncor, Gaithersburg, MD, USA), in order to show specifically apoptotic cells. The ratio of apoptotic and mitotic cells was determined. Statistics was performed using the x^2 method. In order to determine HCHO levels, the culture medium of the untreated and Me-Asc-treated cells was collected 48 hours after treatment.

***Table 1.* Level of HCHO in the culture medium of PC-3 prostate cancer cells 48 hrs after treatment with 100 µg/ml of Me-Asc**

Treatment	*HCHO (mg/ml)*	*% of control*
Control	2.2 ± 0.1	100
Me-Asc	3.6 ± 0.3	164

Results

Biochemical studies

Table 1 showes that a measurable amount of HCHO was found in the culture medium of PC-3 prostate cancer cells. The application of Me-Asc to these cells increased considerably the level of HCHO in the culture medium *(Table 1).*

Biological studies

Effects of Di and Me-Asc on the proliferation of PC-3 cells *invitro* is indicated on *Table 2.* The cell number and viability of the control and the Di treated cells increased steadily (not exponentially because of lack of serum) and did not differ from each other. However, the number of the Me-Asc-treated cells was significantly lower than that of the control even at 24 hours after treatment. This difference increased at 48 hours and was practically unchanged

***Table 2.* Effect of methyl-ascorbigen (Me-Asc), Dimedone (Di) and Me-Asc+Di on the proliferation of PC-3 human prostate cancer**

Treatment	24	48	72
Control	9.2 ± 0.1	13.6 ± 0.4	15.3 ± 0.9
Me-Asc	5.8 ± 0.1*	4.7 ± 1.5**	7.5 ± 1.2***
Di	9.4 ± 1.2	14.2 ± 2.2	17.8 ± 9.3
Me-Asc + Di	7.1 ± 1.7	9.9 ± 2.5****	9.0 ± 1.2
viability			
Control	91 ± 5	99 ± 2	94 ± 3
Me-Asc	88 ± 4	71 ± 6	65 ± 2
Di	94 ± 6	98 ± 1	96 ± 6
Me-Asc + Di	84 ± 2	88 ± 0	88 ± 2

Dose: Me-Asc: 100 µg/ml; Di: 10 µg/ml
Hours after treatment: cell number and viability (V)
* $p<0.005$ vs control
** $p<0.0025$ vs control
*** $p<0.01$ vs control
**** $p<0.01$ vs Me-Asc

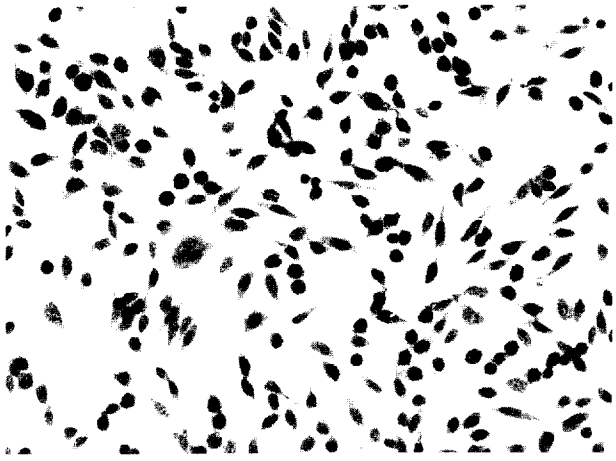

Figure 2. Untreated control PC-3 culture at 48 hours of treatment. HE x 200.

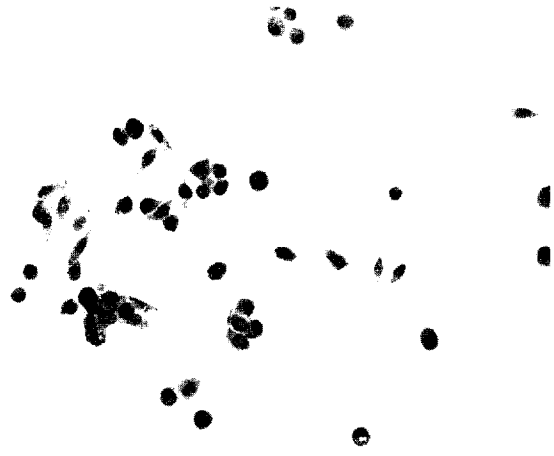

Figure 4. PC-3 culture treated with Me-Asc for 48 hours. Note the high number of pyknotic, shrunken (apoptotic) cells. HE x 200.

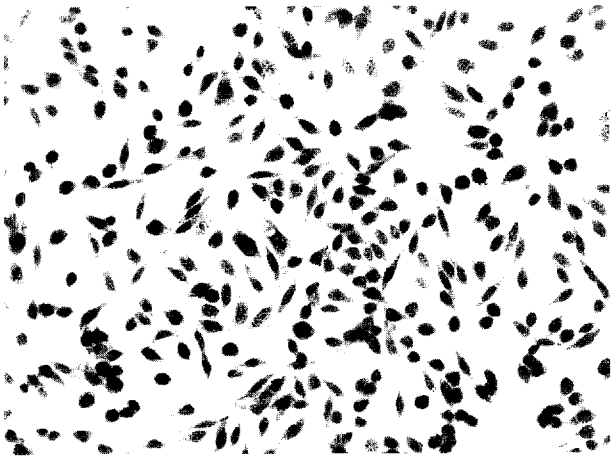

Figure 3. PC-3 culture treated with Di for 48 hours HE x 200.

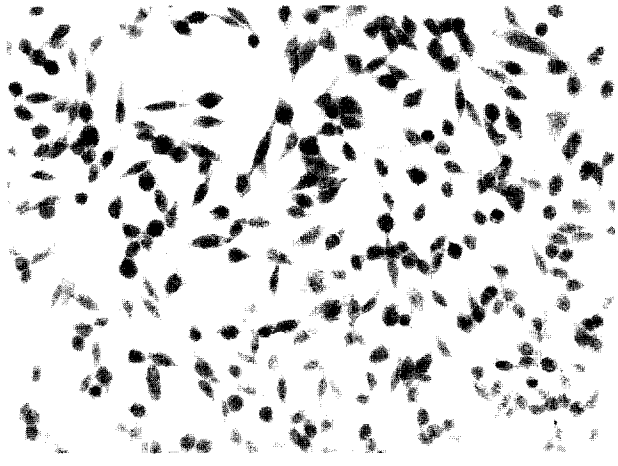

Figure 5. PC-3 culture treated with Me-Asc and Di for 48 hours. Most of the cells appear to be normal HE x 200.

at 72 hours. Combination treatment with Me-Asc and Di resulted in a moderate decrease in cell number which was not significant when compared to the control. Significant difference in cell number between the Me-Asc and the Me-Asc + Di-treated cultures was found at 48 hours after treatment.

The morphology of the control and treated cells, studied 48 hours after treatment, showed practically no differences between the control and the Di-treated cells *(Fig.2. and 3.)*. However, the Me-Asc treated cultures were notably altered. The alteration affected nearly 50% of the cells, which – according to their morphological changes (karyopyknosis, shrinkage of the cytoplasm. Apop-Tag positivity) – underwent the process of apoptosis *(Fig.4)*. The cultures which were treated with both Me-Asc and Di were much less altered *(Fig.5)*. The ratio of apoptotic cells was about 5% *(Table 3)*.

Mitotic figures were present in all cultures, but significantly decreased in number after Me-Asc treatment. Combination treatment resulted in a less significant decrease in mitotic ratio *(Table 3)*.

The immunocytochemical staining with Apop-Tag selectively marked the apoptotic cells. This staining pro-

Table 3. **Apoptotic and mitotic ratio of PC-3 cells after 48 hr of methyl-ascorbigen (Me-Asc), Dimedone (Di), and Me-Asc+Di treatment**

Treatment	*Apoptotic ratio (%)*	*Mitotic ratio (%)*
Control	1.1 ± 0.2	3.1 ± 0.3
Me-Asc	45.0 ± 5.2*	0.6 ± 0.2**
Di	1.0 ± 0.1	3.2 ± 0.3
Me-Asc + Di	5.0 ± 1.1	2.5 ± 0.2***

* p<0.001 vs control; ** p<0.01 vs control; *** p<0.05 vs control

vides a good basis for assessing the apoptotic ratio and made sure that the apoptotic cells assessed by HE staining were genuinely representing apoptosis.

The above results indicate that the profound effect of Me-Asc on cell proliferation and cell death was significantly decreased by simultaneous treatment with Di.

Discussion

According to recent investigations, HCHO is formed within the cell by demethylation (via demethylases and special peroxidases)[21,22] and methylation reactions.[23] These processes can be observed to various degrees during ontogenesis in all biological systems.[24]

The measurable HCHO level is dramatically elevated in virus or other microbially infected tissues (biotic stress), in case of heat shock or alternative stress, and after the application of different exogenous substances (abiotic stress). At the same time, the level of different methylated compounds, which are potential HCHO generators, is considerably decreased.

Our present results indicate that Me-Asc, an exogenous potential HCHO generator, is metabolised. The increased level of HCHO in the culture medium is believed to be only a portion of the HCHO formed in the cells, that is, the detected molecules are only markers for HCHO-forming enzymatic processes in the cells.

Di, a known capture molecule that forms formaldemethone with HCHO, can be used as a reagent for the determination of level of HCHO in a solution.[17] On the other hand Di may capture the HCHO formed in situ, thus may inhibit its biological effect. We suppose, that in our experiment the effect of Me-Asc was prevented by Di via the capture of newly formed HCHO.

The formation of HCHO from different endogenous and exogenous compounds in the cells is a well-known demethylation process,[25-27] and can act as a cell proliferation retardation factor or a cytotoxic substance.[28] Therefore, the antimitotic effect of HCHO from Me-Asc was a predictable result. In addition, HCHO has concentration dependent mutagenic and carcinogenic effects in experimental animals.[29,30]

HCHO is known to cause DNA-protein cross-links. The covalent reactions of HCHO with macromolecules are generally accepted as the cause of its toxic effects.[29] As a very reactive molecule, HCHO can participate in the degradation of DNA which could explain the function of HCHO in the apoptotic process. Apoptosis, programmed cell death, is a physiological process, in which the dying cell plays an active part in its own destruction. It has an important role in the regulation of the balance between cell proliferation and cell death. Defective regulation of apoptosis may play a part in the aetiology of cancer, AIDS, autoimmune diseases and degenerative diseases of the central nervous system.[31] Further, the pharmacological manipulation of apoptosis offers new possibilities for the prevention and treatment of various diseases. The morphological and biochemical characteristics of apoptosis have been thoroughly studied over the past two decades.[32] Apoptosis is obviously under the control of specific genes. Bcl-2 directly regulates apoptosis of B lymphocytes suppressing cell death, helping the cells to survive after injury.[33] B lymphocytes that overexpress bcl-2, have a higher concentration of the bcl-2 protein and survive longer in culture after growth factor deprivation. These cells are resistant to ionizing radiation and glucocorticoids. Also, bcl-2 protects the cells from c-myc induced apoptosis.[34] On the contrary, the protein product of the p53 tumor suppressor gene induces apoptosis.[35]

Many endogenous and environmental influences and injuries can induce apoptosis, which is especially important during embryonic development[36] and in immunological conditions (immune tolerance, immune response, AIDS and perhaps in autoimmune diseases).[37,38]

In this context it is very interesting that in the "real" apoptosis (the fall of autumn leaves from trees) the level of HCHO increases dramatically before leaf falling (e.g. compared to the summer HCHO level, autumn HCHO levels are 5-10 thousand % higher), and at the same time the amount of N-methylated compounds such as choline, trigonelline etc. decreases in correlation with the HCHO formation.[24] In studies of the various types of methylated compounds, very little attention has been paid to the metabolic and genotoxic roles of cellular HCHO. However, the nonspecificity of HCHO binding and the wide variety of possible HCHO acceptors make it difficult to ascertain the actual *in vivo* role of HCHO-yielding reactions in different cells, notably cancer. In spite of these facts, some molecules (e.g. morphine, nicotine, adrenaline, ephedrine[25] are known to demonstrate various biological activities through their methyl groups which are HCHO precursors. Our finding is the first to reveal that the formation of HCHO from the N-methyl group of Me-Asc is linked to its mitotic and apoptotic effects through as yet unknown reactions.

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