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LATENT MEMBRANE PROTEIN-1 OF EPSTEIN — BARR VIRUS INCREASES SENSITIVITY TO ARSENIC TRIOXIDE-INDUCED APOPTOSIS IN NASOPHARYNGEAL CARCINOMA CELL

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Background and Aim: **Epstein — Barr virus (EBV)-encoded LMP1 is suggested to have an important role in the pathogenesis and development of nasopharyngeal carcinoma (NPC). Our previous study showed that As_2O_3 exhibited growth inhibition of NPC in animal model. Here, we further explore whether LMP1 is involved in As_2O_3 anticancer effects in NPC cell line.** ***Methods:*** **Both the stable expressing LMP1 cell line HNE1-LMP1 and its parental cell line HNE1 without LMP1 expression were used as *in vitro* models to assess arsenic trioxide effect. Both cell lines were treated with As_2O_3 for 72 h. The median inhibition concentration (IC_{50}) was assessed by the MTT assay. Apoptosis was observed by phase-contrast microscopy and TUNEL staining. The alteration of telomere lengths was detected by Southern blotting.** ***Results:*** **IC_{50} for As_2O_3 in HNE1-LMP1 cells and HNE1 cells was 2.22 and 5.09 μmol/L, respectively. After exposure to 2 and 4 μmol/L As_2O_3 for 72 h, the apoptotic index in HNE1-LMP1 was 26.27 ± 1.3 and 49.13 ± 1.4%, respectively. On the contrary, in HNE1 cells the apoptotic index was 12.6 ± 0.9 and 33.20 ± 1.3%, respectively. As compared with parental cell line HNE1, HNE1-LMP1 cells were more sensitive to growth inhibition and apoptosis ($p < 0.001$). The elongation of telomere length was also found in HNE1-LMP1 cells. Meanwhile, longer telomeres in HNE1-LMP1 cells failed to maintain telomere stabilization, instead, it prone to be shortened when exposure to As_2O_3, as comparing with HNE1 cells.** ***Conclusion:*** **LMP1 plays important role in enhancing NPC cell response to As_2O_3. The elongation of telomere length induced by LMP1 may contribute to the mechanisms of As_2O_3 sensitivity.**

Key Words: **latent membrane protein-1, nasopharyngeal carcinoma, arsenic trioxide, telomere.**

Nasopharyngeal carcinoma (NPC) occurs sporadically in the West but is endemic in southern China. The reported incidence is between 15 and 50 per 100,000 [1]. Up to now, radiotherapy or combined radiotherapy and chemotherapy are the most efficient strategies in NPC treatment, which cures 80–90% of cases presenting with early stage tumor [1]. However, the outcome of patients with advanced stage disease at diagnosis or relapsing after first-line therapy is poor [1]. Thus, additional forms of effective, low-toxicity treatment are warranted. Previously, we reported that arsenic trioxide (As_2O_3) may be a potential medicine for human NPC treatment [2]. We found that NPC xenografts of CSNE-1 cells exhibited differentiation and apoptosis after As_2O_3 treatments at a dose of 5 mg/kg and little toxicity was observed in the experimental animals. We also disclosed that As_2O_3 can sensitize human NPC cancer cells to ionizing radiation *in vitro* [3].

In view of the important role of latent membrane protein-1 (LMP1) inhibiting differentiation and apoptosis of NPC [4, 5], we think it is worthwhile to determine whether LMP1 modifies As_2O_3 induced apoptosis in NPC cells in order to better understand the molecular mechanisms of As_2O_3 action. To answer this issue, an artificial stable expressing LMP1 cell line HNE1-LMP1 was used in the study, and its parental cell HNE1 was used as control.

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Abbreviations used: As_2O_3 – arsenic trioxide; EBV – Epstein – Barr virus; LMP1 – latent membrane protein-1; NPC – nasopharyngeal carcinoma; TRF – terminal restriction fragment.

MATERIALS AND METHODS

Cell and culture condition. HNE1 originating from a poor-differentiated NPC had lost EB virus with a long-term *in vitro* passage (over 100 passages) [6]. HNE1-LMP1 capable to express constantly LMP1 has been obtained upon LMP1 cDNA transfection into HNE1 cell line. Both cell lines were established at the Cancer Research Institute of Hunan Medical University (China) and were kindly provided by Prof. Y. Cao. The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated newborn calf serum at 37 °C in a humidified CO_2 incubator containing 5% CO_2 and 95% air.

Reagents. As_2O_3 was obtained from Harbin-Eda Pharmaceutical Co. Ltd, China. Monoclonal antibodies to LMP1 were purchased from DAKO, Denmark, and Telomere Length Assay Kit was obtained from Roche Molecular Biochemicals, Indianapolis, IN, USA.

Experimental design. Cells were firstly cultured with different concentration of As_2O_3 (1–5 μmol/L) for 72 h to detect the inhibition rate. Then we select As_2O_3 at the concentration of 2 and 4 μmol/L as study points to observe its effects on apoptosis and the alteration of telomere length in both cell lines.

MTT assay. The cells were seeded onto 96-well culture plates (3×10^3 cells in 190 μl of medium per well) and then were cultured at 37 °C in 5% CO_2 and

> 95% humidity for 24 h. Different concentrations of As_2O_3 in RPMI-1640 were added to the wells to final concentration of 0 to 5 μmol/L. The cells were cultured for another 72 h. Then, 20 μl of 5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well and the cells were cultured for 4 h. Finally, the culture medium was discarded, and 150 μL DMSO was added to each well and the absorbance at 492 nm (A_{492}) was measured with microplate reader. Inhibition rate was calculated by the formula:

$$\frac{1 - A_{492} \text{ in treatment group}}{A_{492} \text{ in control group}} \times 100\%.$$

The concentration of 50% growth inhibition (IC_{50}) was calculated.

Phase-contrast microscopy. Cells treated with As_2O_3 in 25 ml culture flasks were examined under phase-contrast microscopy.

Apoptosis detected by TUNEL (TdT-mediated dUTP nick end labeling) assay. TUNEL method was performed for detection and quantification of apoptotic cells by use of *in situ* cell death detection Kit (Boehringer, Mannheim) according to instructions of manufacturer. Cells in which the nuclei or cytoplasm were dyed dark purple were identified as those undergoing apoptosis. Five visual fields for each specimen and 100 nuclei in each visual field were observed. The average ratio of apoptotic cells to the total number of cells counted was expressed as apoptotic index (AI, %).

Telomere length assay. Genomic DNA samples were prepared as described in [7]. Cells were lysed and proteins were digested in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K at 48 °C overnight. Following two extractions with phenol and one with chloroform, DNA was precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE). Telomere length was detected using TeloTAGGG telomere length assay according to the manufacturer's protocol. For each sample, 1 μg of genomic DNA was digested with Rsa I/Hinf I (Sigma), separated on a 0.8% agarose gel, transferred to a nylon membrane Hybond-N^+ (Amersham, England), and hybridized with a telomere specific digoxigenin (DIG)-labeled probe, incubated with anti-DIG-alkaline phosphatase and detected by chemiluminescence. The blotted signal was divided into 30 equidistant intervals from 1.9 to 21.2 kilobases to calculate mean telomere length (terminal restriction fragment, TRF) using the formula TRF = Σ (ODi)/Σ (ODi/Li), where ODi was the chemiluminescent signal and Li was the length of the TRF fragment at a position [8].

Statistical analysis. All experiments were performed in triplicate. The Student's *t*-test was used to determine the statistical significance of the data obtained. The software used was SPSS10.0 for Windows. $P < 0.05$ was taken to represent a statistically significant difference between the groups.

RESULTS

Proliferation retardation effect in NPC cells. As shown in Fig. 1, As_2O_3 produced more pronounced proliferation inhibition in HNE1-LMP1 cells than in HNE1 cells ($P < 0.001$). The median inhibition concentration (IC_{50}) at 72 h for HNE1-LMP1 cells was 2.22 μmol/L *vs.* 5.09 μmol/L for HNE1 cells. This result indicates that HNE1-LMP1 cells were more susceptible to As_2O_3-induced cell death and showed 2.3-fold increased sensitivity to As_2O_3 compared to HNE1 cells.

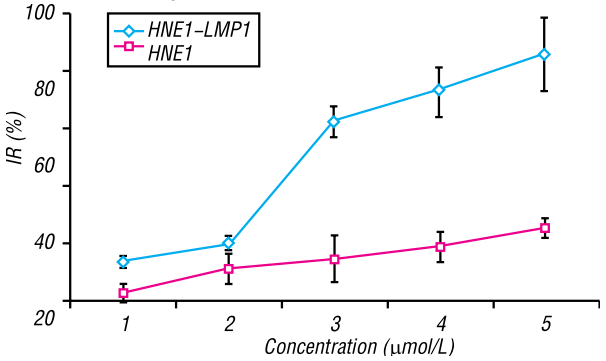


Fig. 1. Inhibition of NPC cell proliferation by As_2O_3

Morphology of living cells. Cells without As_2O_3 treatment appeared polygonal with cell-to-cell attachment. In parallel studies, in the group of As_2O_3 treatment, the cells had shrank significantly and displayed a ball-like appearance with loss of cell-to-cell contact, and significant changes of cell morphology could be observed up in HNE1-LMP1 cells (Fig. 2). Furthermore, the patterns of cell death were determined by TUNEL staining. The results are shown in Fig. 3. After 0, 2, 4 μmol/L As_2O_3 treatment for 72 h, AI was 3.3 ± 0.7, 26.27 ± 1.3 and 49.13 ± 1.4%, respectively in HNE1-LMP1, whereas it was 3.5 ± 0.9, 12.6 ± 0.9 and 33.20 ± 1.3% in parental line, respectively. Obviously, there was statistically different apoptotic response between the two cell lines ($p < 0.001$).

The alteration of telomere length. By TRF analysis, the telomere length of the HNE1-LMP1 cells was elongated as compared with its parental cells (4.9 *vs* 4.3 kb). Interestingly, when the cells were exposed to 2 and 4 μmol/L As_2O_3, TRF length was shortened significantly. On the contrary, the telomere length in HNE1 cells changed little after As_2O_3 treatment. The telomere length assay was repeated three times and the results of all experiments showed the same pattern of telomere shortening. Results of one representative experiment are shown in Fig. 4.

DISCUSSION

At present time, abundant evidence shows that As_2O_3 is a novel broad-spectrum anticancer agent not only for acute promyelocytic leukemia [9], but also for solid cancers. It can induce apoptosis in many solid cancer cell lines, including esophageal carcinoma [10], gastric cancer [11], NPC [2], lymphoid malignancies [12], gynecological cancers [13], prostate cancer [14] and multiple myeloma [15]. Nevertheless, susceptibility of cancer cells to As_2O_3-induced apoptosis may be affected by various factors such as

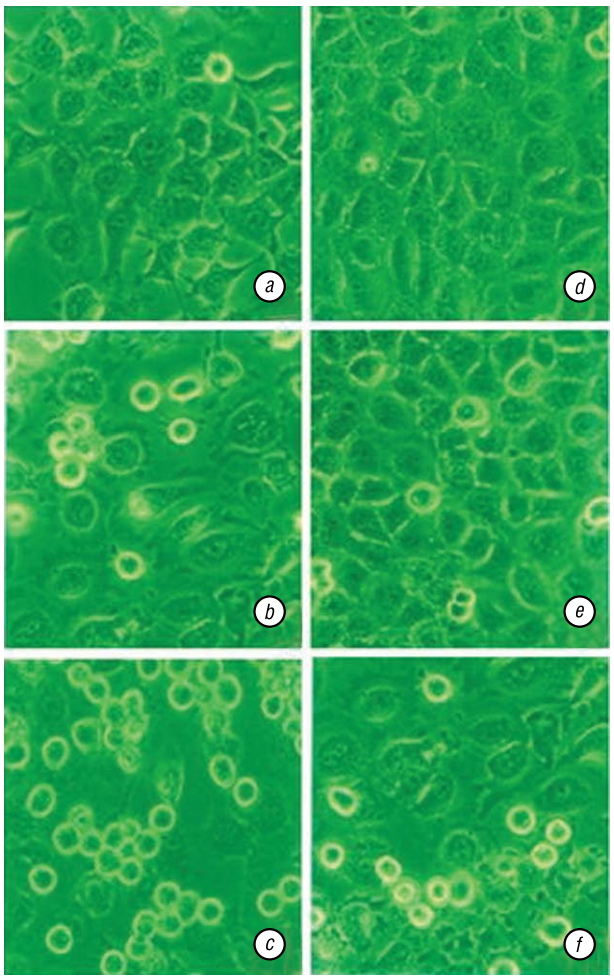


Fig. 2. Effect of As_2O_3 on cell morphology. Photos were taken under 200 × magnification using a phase contrast microscope. *a*: before As_2O_3 was added, the HNE1-LMP1 cells showed a single layer of squamous appearance with gaps between cells; *b*: after exposure to 2 μmol/L As_2O_3 for 72 h, the HNE1-LMP1 cells became shrunken, round shaped and dissociation cellular attachment occurred; *c*: after exposure to 4 μmol/L As_2O_3 for 72 h, mostly floating HNE1-LMP1 cells were observed; *d*: before As_2O_3 was added, the HNE1 cells showed a single layer of squamous appearance with gaps between cells; *e*: after exposure to 2 μmol/L As_2O_3 for 72 h, few HNE1 cells became shrunken, round shaped and cellular attachment remained; *f*: after exposure to 4 μmol/L As_2O_3 for 72 h, moderated floating HNE1 cells were observed

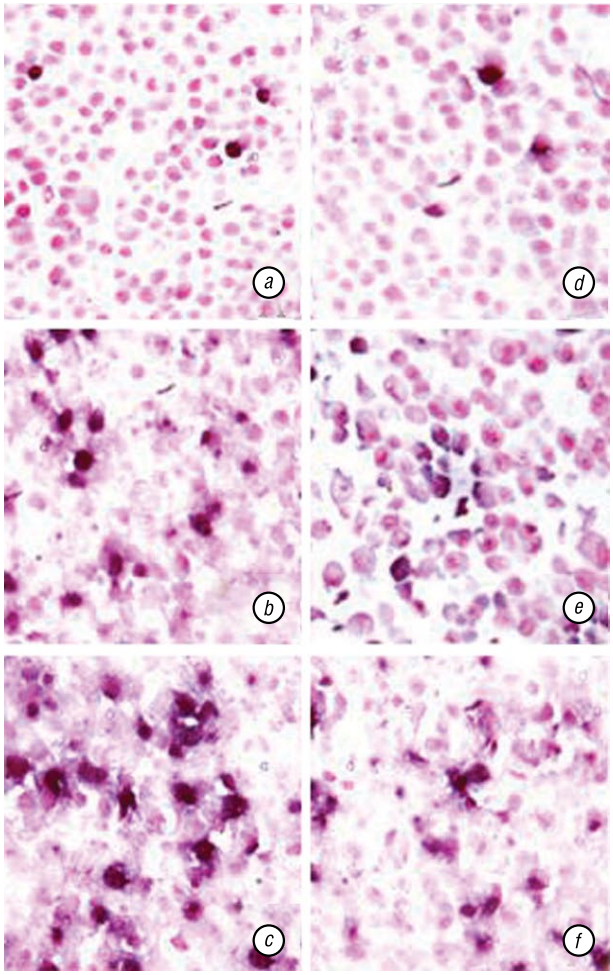


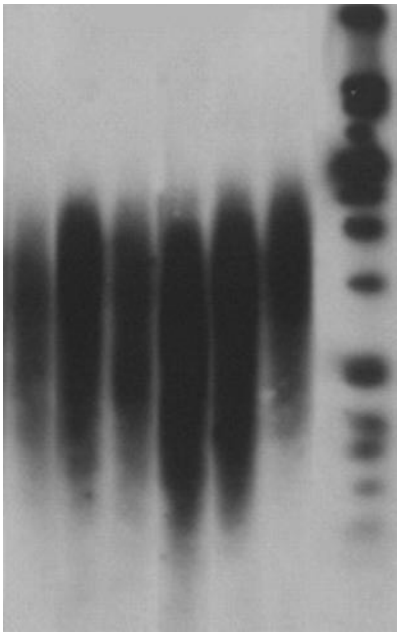
Fig. 3. TUNEL analysis of apoptotic cells. Cells in which nucleus or cytoplasm was dyed dark purple were identified to be undergoing apoptosis (200 × magnification). *a*: untreated HNE1-LMP1 cells; *b*: HNE1-LMP1 cells exposure to 2 μmol/L As_2O_3 for 72 h; *c*: HNE1-LMP1 cells exposure to 4 μmol/L As_2O_3 for 72 h; *d*: untreated HNE1 cells; *e*: HNE1 cells exposure to 2 μmol/L As_2O_3 for 72 h; *f*: HNE1 cells exposure to 4 μmol/L As_2O_3 for 72 h

cellular reductive oxidative state, telomere state, and intracellular GSH content, etc. For instance, buthionine sulfoximine sensitized hepatocellular carcinoma cell lines to As_2O_3 by depleting GSH contents [16].

Recent evidence also suggests that diethyloxadicarbocyanine increases sensitivity to arsenic trioxide-induced apoptosis by altering telomere state [17]. Our present study further provides evidence that LMP1 is another factor to influence sensitivity of NPC cells to As_2O_3. LMP1-transfectants showed 2.3-fold increased sensitivity to As_2O_3 judging by IC_{50} values and about two-fold increase in AI as compared with parental cell line.

LMP1, an important protein encoded by EBV, is an integral membrane protein, which acts like a constitutively active receptor, and confers a survival advantage to EBV infected cells by protecting them from apoptosis, and this was shown to depend on LMP1-induced up-regulation of the antiapoptotic protein Bcl-2, Mcl-1, A20 [18]. Furthermore, in cultured cell line, heterologous expression of LMP1 leads to inhibition of terminal differentiation [5]. Moreover, growth characteristics of NPC tumors have been correlated with LMP1 expression levels. Detectable LMP1 protein is linked with the expression of EGFR and Ki67 in NPC biopsies [19], and LMP1-positive NPC tumors appear to grow faster and more expansively than LMP1-negative NPC tumors [20].

Nevertheless, several studies demonstrate that the expression of LMP1 correlates with favorable outcome of chemotherapeutic treatment in patients with Hodgkin's disease [21]. Besides, the expression of LMP1 also correlates with a better prognosis of NPC in a Chinese population; in contrast, LMP1-negative NPCs recurred at a higher frequency and had an increased tendency to metastasize [20]. Our findings further provide direct evidence that LMP1 is capable of promoting some NPC-derived cell line HNE1 response to As_2O_3. The mechanisms of LMP1 sensitizing NPC cells to DNA damaging agents are far from being clear. Mitochondrial discharge of cytochrome *c*, a co-factor required for activation of downstream cas-



No. of experiment	Mean telomere length in each lane (Kbp)					
	1	2	3	4	5	6
1	4.9	4.4	3.7	4.3	4.2	4.0
2	4.9	4.4	3.6	4.3	4.2	4.1
3	4.9	4.3	3.7	4.2	4.1	4.1

Fig. 4. Alteration of telomere length. The upper part indicates the mean telomere length (terminal restriction fragments, TRF) in HNE1-LMP1 cells and HNE1 cells by telomere length assay. The lower part indicates the calculated value of mean telomere length in each lane from three typical experiments. Values shown in experiment 1 reflect the result shown in the upper part figure. Lane 0: molecular marker; Lane 1–3: HNE1-LMP1 cells treated by As_2O_3 for 72 h (0, 2, 4 μmol/L); Lane 4–6: HNE1 cells treated by As_2O_3 for 72 h (0, 2, 4 μmol/L)

pases, was shown to be potentiated upon expression of LMP1 in the cells [22]. Besides, other factors such as inducing micronucleus formation, repressing DNA repair, interfering with the balance of Bcl-2 and Bax were also thought to participate in LMP1 enhancing apoptosis effect mediated by the chemotherapeutic agents [23, 24].

Telomeres, unique DNA-protein structures, which contain noncoding TTAGGG repeats and telomere-associated proteins, are normally shortened with each cell division. When the telomeres are shortened to a critical length, the signal for senescence or cell apoptosis is activated. Telomerase activity is necessary for maintenance of telomere stability. LMP1 was reported to take important part in up-regulating telomerase activity [25]. Previously, we have shown that arsenite suppressed telomerase activity in CSNE-1 xenografts, NPC cell line with natural expression of LMP1 [26]. We further investigate the relationship between alteration of telomere length and LMP1 status. Our results showed that As_2O_3 induced telomere shortening in dose-dependent manner. Except for the suppression of telomerase activity, As_2O_3 has also been reported to intercalate into base pairs of DNA and generate toxic oxygen free radicals, which cause single-or double-strand breaks in DNA and contribute to the telomere shortening. As_2O_3 is a well-known apoptosis inducer. Many possible modes of arsenic action have been proposed, including altering the function of several enzymes and signaling molecules, eliciting mitochondrial perturbation with release of apoptogenic factors (cytochrome *c* and others) from these organelles, influencing DNA repair and DNA methylation patterns [10–15]. Here, we further provide direct evidence that As_2O_3 affects telomere length in the concentration-dependent manner, which may be associated with cell apoptosis. Because telomere loss is associated with activation of apoptotic pathway and growth arrest, the mechanisms of cell growth retardation and apoptosis in the current study after As_2O_3 treatment were guessed, at least in part, is associated with telomere loss.

Another interesting feature of this study is the discrepancy of telomere length in two cell lines. As stated in the results, HNE1-LMP1 cells showed some elongation of telomere length as compared with the parental cells revealed by the TRF analysis. The negative relation between telomere length and apoptosis has been reported [27]. Therefore, it was a surprise for us to observe that the elongated telomeres of HNE1-LMP1 cells fail to protect the cells from apoptosis induced by As_2O_3. On the contrary, more pronounced telomere shortening with accompanying apoptosis occurred in HNE1-LMP1 cells after As_2O_3 in concentration-dependent manner, whereas parental HNE1 cells experienced rather moderate alteration in apoptosis and telomere length. In fact, our data seem to be consistent with the data of other authors indicating that longer telomeres can exacerbate cancer cells sensitivity to hydrogen peroxide [28].

Arsenic exerts its toxicity in part by generating reactive oxygen species, which consequently deranges cellular signaling pathways and causes DNA strand breaks as well as chromosome damage [29]. Liu, et al. reported that arsenic-induced oxidative stress promotes telomere attrition and apoptotic cell death in mouse embryos [30]. In present study, we speculate that LMP1-mediated As_2O_3 sensitivity may be, at least in part, associated with the shifts in telomere length. Indeed, a similar result has been observed in cell line 293 where longer telomeres provide for increased sensitivity to cell growth inhibition and apoptosis induced by As_2O_3 [17].

In summary, this study demonstrates that LMP1 expression enhances vulnerability to As_2O_3-induced apoptosis. The possible reason may be associated with elongation of telomere length after LMP1 introduction into HNE1 cells. In addition, As_2O_3 shortening telomere length was especially evident in LMP1 expressing cells and showed a concentration-dependent manner. This finding may provide some useful information about using As_2O_3 in NPC treatment. Further studies are necessary to find out the exact role of LMP1 in As_2O_3 inducing apoptosis effect.

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ЛАТЕНТНЫЙ МЕМБРАННЫЙ БЕЛОК ВИРУСА ЭПШТЕЙНА — БАРР ПОВЫШАЕТ ЧУВСТВИТЕЛЬНОСТЬ КЛЕТОК НАЗОФАРИНГЕАЛЬНОЙ КАРЦИНОМЫ К ИНДУКЦИИ АПОПТОЗА ПОД ДЕЙСТВИЕМ ТРЕХОКИСИ МЫШЬЯКА

Цель: **белок LMP1, кодируемый вирусом Эпштейна — Барр, играет важную роль в патогенезе назофарингеальной карциномы (NPC). В наших предыдущих исследованиях было показано, что As_2O_3 ингибирует рост ксенотрансплантатов NPC у мышей. Задача исследования состояла в том, чтобы выяснить влияние LMP1 на чувствительность клеток NPC к действию As_2O_3, в частности, на индукцию апоптоза в них.** ***Методы:*** **в качестве моделей *in vitro* использовали линию клеток HNE1 и линию HNE1-LMP1 со встроенным LMP1, которая активно экспрессирует этот белок. Клетки обрабатывали As_2O_3 на протяжении 72 ч. Жизнеспособность клеток определяли с помощью MTT-теста. Апоптоз регистрировали методом TUNEL. Анализ длины теломер выполняли методом Саузерн-блот-гибридизации.** ***Результаты:*** **IC_{50} для As_2O_3 в клетках HNE1-LMP1 и HNE1 составила 2,22 and 5,09 мкмоль/л соответственно. Апоптотический индекс при концентрациях As_2O_3 2 и 4 мкмоль/л составил 26,27 ± 1,3 и 49,13 ± 1,4% соответственно в клетках HNE1-LMP1 по сравнению с 12,6 ± 0,9 и 33,20 ± 1,3% в исходной линии HNE1. В клетках HNE1-LMP1 отмечено увеличение длины теломер, что, однако, не приводило к снижению чувствительности этих клеток к индукции апоптоза.** ***Выводы:*** **клетки HNE1-LMP1 оказались более чувствительными к индукции апоптоза под воздействием As_2O_3 ($p < 0,001$). Длина теломер в клетках со стабильной экспрессией LMP1 увеличивается по сравнению с таковой в исходной линии.**

Ключевые слова: **латентный мембранный белок-1, назофарингеальная карцинома, трехокись мышьяка, теломеры.**