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Incubation with DNase I inhibits tumor cell proliferation

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Deoxyribonuclease I (DNase-I) plays an important role in the elimination of damaged, aging, and cancer cells. Various authors suggest that programmed cell death (PCD) is attenuated in cancer cells due to a reduced activity of DNase-I.

Material/Methods: In this study, cell viability (violet crystal stain), cell proliferation (tritiated thymidine), and DNA degradation of tumor cells (Calu-1, SK-MES-1, HeLa, HEp-2, L-929) incubated with different concentrations of DNase I were evaluated. PBMN cells and human fetal fibroblasts served as controls.

Results: The results showed a >90% decrease in HeLa and HEp-2 and a 50–90% decline in Calu-1, SK-MES-1, and L-929 cell viability when incubated with 9 µg/ml of DNase-I compared with control cells ($p<0.05$). The incorporation of [^{3}H]thymidine showed a 50% decrease in tumor cells. Control cells showed no significant differences. DNA degradation was observed in tumor cells after nuclease treatment; however, the typical DNA ladder characteristic of apoptotic cells was not observed. The morphologies of some of the DNAse-I-treated tumor cells suggested autoschizis.

Conclusions: These results suggest that the use of a DNA nuclease might have some benefits in the treatment of cancer since it inhibits cell growth, probably by inducing autoschizic cell death.

key words: **apoptosis • cell culture • deoxyribonuclease I • endonucleases • HEp-2 cell • HeLa cell • inhibition of cell growth • PBMN cells • tumor cells**

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BACKGROUND

Deoxyribonuclease I (DNase-I) is an endonuclease that cleaves double-stranded DNA and has a crucial role in programmed cell death (PCD) [1]. Other nucleases, such as NUC-18, DNase-II and calcium-independent DNase, are also synthesized during PCD [2]. DNase-I is broadly distributed in the cell and plays an important role in the repair, replication, and degradation of DNA in non-tumor cells [3,4]. It also eliminates ageing and tumor cells [5]. DNase-I treatment prevents blood-born liver metastasis of tumor cells and segregates tumor-cell aggregates, thus facilitating the entry of different cytotoxic agents [6,7]. Likewise, Tokita et al. [8] showed that the intravenous administration of Dnase-I appeared to enhance tumor-cell arrest in the lung microvasculature. Nevertheless, several reports mention that the activity or the concentration of nucleases is altered in tumor cells [9,10].

Probable benefits of the intracellular reactivation of nucleases in the control of cancer have been mentioned by Taper [11,12]. Specifically, the use of nucleases in the treatment of cancer has been suggested by Ben-Yehudah et al. [13] and Linardou et al. [14]; however, nucleases' advantages and efficacy as antitumor agents still need to be corroborated. The aim of this paper was to evaluate the effect of DNase-I treatment in different cancer cells in relation to cell proliferation and viability. Our results demonstrate a reduction in the proliferation of the tumor cells following incubation with the nuclease. Based on morphological data and DNA analysis, we found that our treatment induces significant cell death in the tumor cell lines used, including the type originally described as autoschizis or autoschizic cell death [15–18].

MATERIAL AND METHODS

Cell lines

HEp-2 (human larynx carcinoma), HeLa cells (human cervical carcinoma), Calu-1 (human lung epidermoid carcinoma), SK-MES-1 (human lung carcinoma), and L-929 (mouse connective tissue) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). MRC-5 cells (normal lung tissue of a 14-week-old male fetus) and peripheral blood mononuclear cells (PBMNs) obtained from two healthy voluntaries (PBMN-1 and PBMN-2) served as controls. The cells were grown in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma), 100 IU/ml penicillin, and 100 µg/ml streptomycin (In Vitro, Mexico) at 37°C in a humidified 5% CO_2 atmosphere.

Cell viability

1×10^4 cells per well were seeded in 96-well culture plates (NUNC) for 24 h. After this time the culture medium was discarded and the cells were incubated with 0.75, 1.5, 3, 6, and 9 µg/ml DNase I (donated by Laboratory of Philosophic and Scientific Researchers S.A. of C.V., HYPATIA, México) in 0.1 ml of culture medium for 24 h. Cell viability was evaluated using crystal violet stain. Plates were washed with phosphate-buffered saline (PBS) and stained with 100 µl of a 0.1% crystal violet solution (w/v) (Sigma) in 70% ethanol for 20 min. The bound dye was obtained with 100 µl of 33% acetic acid in PBS for 5 min. Optical density was determined at 595 nm in a plate spectrophotometer (BIO-TEK Instruments Inc., Highland Park, Winooski, VT, USA). Results are expressed in percentages. The mean absorbance obtained from the control cells was considered to be 100%. Experiments were done in triplicate. Crystal violet stain allows the evaluation of cell viability as well as cellular proliferation in relation to the culture time [19].

Cell proliferation

5×10^4 cells were cultured in 96-well plates for 24 h, then incubated for 24 h with the DNase concentrations previously mentioned. The cells were then incubated with 1 µCi/well [^{3}H]-thymidine (specific activity: 86.5 Ci/mmol, Amersham, Buckinghamshire, U.K.) for 18 h. Afterwards a Cell Harvester (Brandel, Gaithersburg, MD, USA) was used to harvest the cells. Cellulose filters were placed in vials with 3 ml of scintillation liquid and read in a beta radiation counter (Beckman LS 6000SE, Fullerton, CA, USA). Results are expressed in percentages. The mean counts per minute (cpm) obtained from the control cells were considered to be 100%. Experiments were done in triplicate.

DNA analysis

Six-well culture plates were used to seed 2×10^6 cells in 1 ml of culture medium for 24 h. At the end of this time, the cells were incubated with DNase I for 6, 12, and 24 h. Afterwards, adherent and suspended cells were recovered. The DNA of both cells, adhered and suspended, was obtained using an Apoptotic DNA-Ladder kit (Roche Diagnostics, Mannheim Germany) and analyzed in a 1% agarose gel running at 75 V for 1.5 h. DNA was stained with 5 µl of ethidium bromide (stock solution 50mg/5ml) and visualized in a UV transilluminator ULTRA•LUM (Carson, CA, USA).

Statistical analysis

STAT v. 1.3 software was used for Tukey and Dunnett comparative tests. Results are reported as the mean ± standard error of the mean. Statistical significance was set at $p<0.05$.

RESULTS

Cell viability

Our results showed a dramatic diminution in the percentage of cell viability when a 9 µg/ml concentration of DNase-I was used. HEp-2 and HeLa cells were the most affected (diminution of 95%), while the other cell lines showed a fall in their viability of nearly 50% (Figure 1A). PBMN-1 and -2 and MRC-5 cells did not have significant modifications in cell viability (Figure 1B). Culture of tumor cells with nuclease for 6 and 12 h did not show significant morphological changes; it was necessary to incubate the cells for 24 h with DNase-I to observe effects of nuclease on the tumor cells. Incubation with only the DNase solvent had no effect on the cells' viability (Figure 1C).

Cell proliferation

A DNase-I concentration of 9 µg/ml inhibited more drastically the incorporation of tritiated thymidine (Figure 2A).

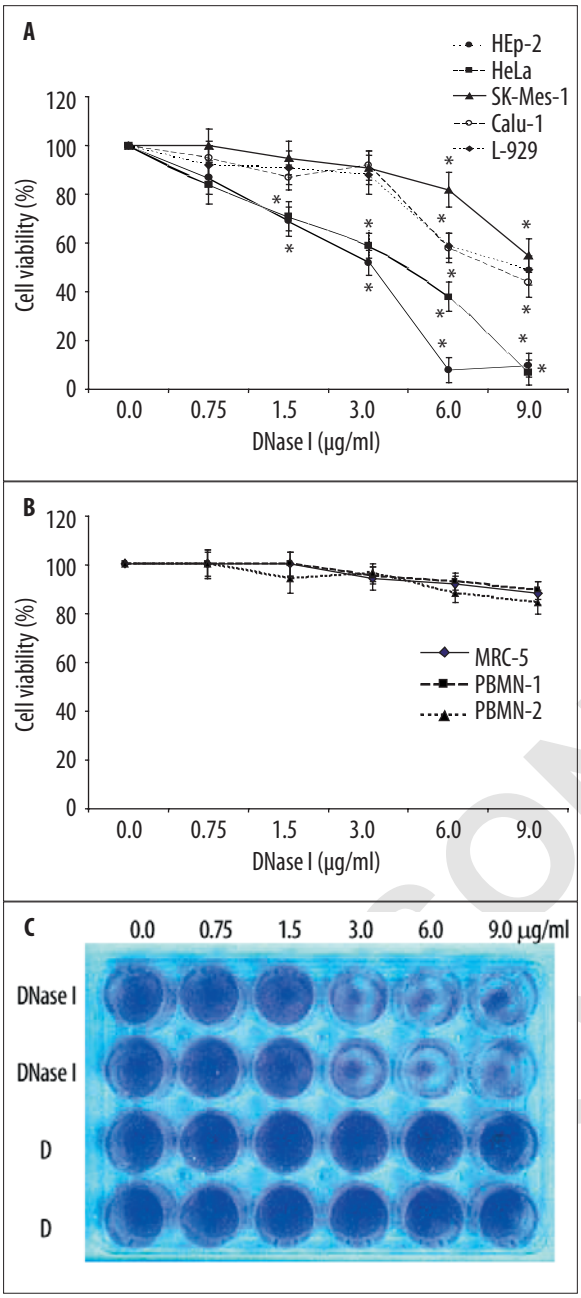

Figure 1. Cell viability of tumor (**A**) and control (**B**) cells, incubated with DNase-I for 24 h at 37°C in a 5% CO_2 humid atmosphere and evaluated by crystal violet staining. Results are expressed as the mean ± standard error of the mean. *$p<0.05$. (**C**) shows the crystal violet staining of HEp-2 cells incubated with different concentrations of DNase-I or with the solvent only D, which was as an internal negative control.

The reduction was 34% in SK-Mes cells, 38% in Calu-1 cells, 50% in HeLa cells, 52% in L-929 cells, and an important 58% in HEp-2 cells. Control cells did not show statistically significant variations (Figure 2B).

Our results also showed morphological differences in the cells incubated with DNase-I. Figure 3A shows HEp-2 cells incubated with 9.0 µg/ml DNase-I for 24 h. These cells lost

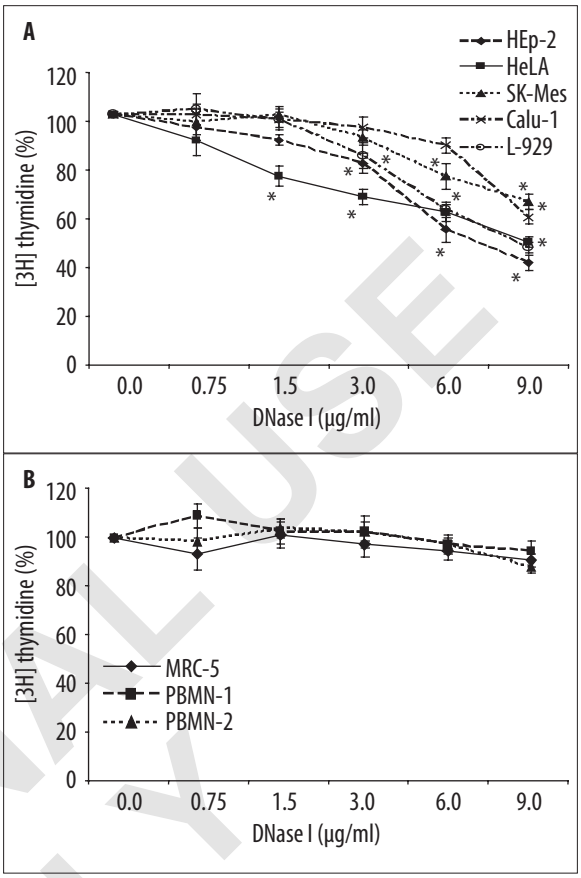

Figure 2. Thymidine incorporation of tumor (**A**) and control (**B**) cells, incubated with DNase-I for 24 h at 37°C in a 5% CO_2 humid atmosphere. Cells were incubated with 1 µCi/well of [^{3}H]-thymidine (specific activity 86.5 Ci/mmol). Results are expressed as the mean ± standard error of the mean. *$p<0.05$.

cell morphology, determined by a decrease in cell volume and manifested by cell rounding, nuclear collapse, vesicle formation in the cellular membrane, and cell detachment. Similar results were observed in the other tumor cells studied (data not shown). HEp-2 cells cultured in the solvent vehicle are shown in Figure 3B. Our results did not show morphological changes in these cells.

DNA analysis

Although we observed degradation of DNA (mainly in HeLa and HEp-2 cells), it was not possible to observe the DNA ladder described in cells that are in apoptosis (data not show). Our results only showed an insignificant degradation of DNA in control cells (data not show).

Discussion

There is experimental evidence demonstrating inhibition or activity reduction of apoptotic nucleases in tumor cells [6]. Roger Daoust [20] showed a deficient activity of DNases in isolated cells taken from more than 60 different types of tumors. Likewise, Mély-Goubert et al. [21] showed the inhibition of DNase-I in a tumor cell line. Alternatively, increased DNase activity is associated with a better response to antitu-

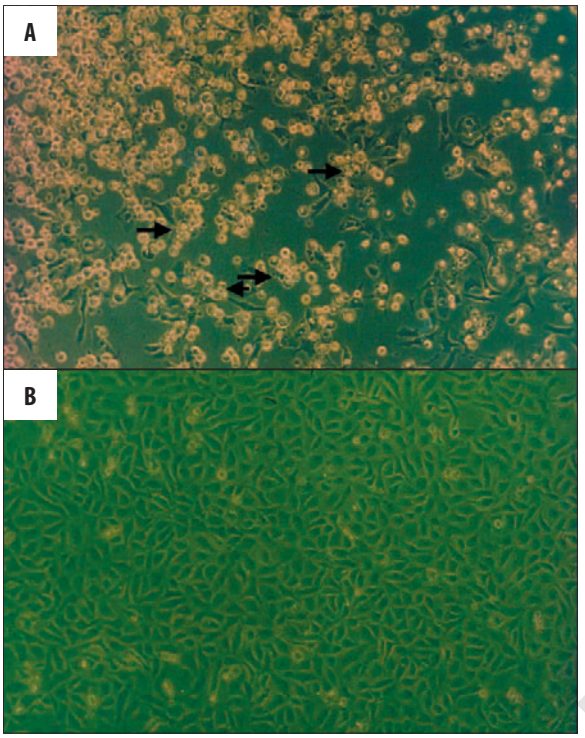

Figure 3. Morphology of HEp-2 cells. The upper photograph (**A**) shows the modifications in cell morphology, including cell shape, cell rounding, cell blebbing, and in some cells (dark arrows) probable autoschizis can be seen. The lower photograph (**B**) shows HEp-2 cells incubated with the vehicle used to resuspend the DNase-I, which we used as an internal negative control in all our experiments. The morphology changes seen in HEp-2 cells are representative of the modifications observed in all the other tumor cell lines (data not shown) (40× amplification).

mor therapy in patients with cancer [11,12,22]. Preliminary results obtained from patients treated with DNase-I plus the conventional cytotoxic agents showed a better response after this treatment [23]. However, the action mechanism of DNase is still unclear.

Sugihara et al. [9] and Tokita et al. [10] observed inhibition of metastasis or inhibition of tumor formation in the presence of DNase-I; nevertheless, the authors did not evaluate cell viability. Our results showed a drastic fall in cell viability in tumor cells after incubation with 9.0 µg/ml DNase-I. This effect is probably linked with the high proliferative capacity of the tumor cells. Cancer cells with a great mitotic activity are more sensitive to the action of different cytotoxic agents as a consequence of the absence of a nuclear membrane, resulting in continuous exposure of nuclear DNA to drugs. Although we did not evaluate the entrance of DNase into the cell, nucleases damaged the *di novo* DNA being formed in non-tumor cells with a high proliferative capacity [24,25]. The ability of nucleases to affect the latter in tumor cells can be used therapeutically. The entrance of a minimum quantity of DNase-I into the tumor cell is required to begin the apoptotic process [26,27]. Damaged apoptotic cells, or tumor cells, have enhanced cell membrane permeability [28], which probably eases the entrance of DNases.

Although cells in apoptosis show a DNA ladder which derives from the activity of DNase-I, our analysis of DNA did not show such DNA ladder. However, it has been reported that some cell types undergoing apoptosis do not show the DNA ladder, despite the fact that DNase is active. Another possibility is that a tumor cell incubated with the nuclease is in autoschizis. This is a novel type of necrosis characterized by minimal condensation of heterochromatin, a decrease in cell volume, cell rounding, cell blebbing, progressive nuclear shrinkage, progressive loss of organelle-free cytoplasm, and random DNA cuts by DNase [15–18]. We observed that HEp-2 cells incubated with DNase-I for 24 h showed some of the characteristics that define autoschizis. Additionally, our experiments revealed that the culture of tumor cells with the nuclease for 6 and 12 h did not show significant morphological changes; it was necessary to incubate the cells for 24 h with DNase-I to be able to observe effects of the nuclease on the tumor cells. This is probably due to the need of the cell to accomplish cellular DNA duplication where the nuclear membrane is absent, a fact that facilitates the action of nucleases on the tumoral DNA.

It was clear that not all the cell lines responded equally to the DNase treatment. This difference is possibly secondary to the presence of different actin mutants, since recent evidence shows the important role of actin in the regulation of the apoptotic process in tumor cells [29].

Our results showed a gradual reduction in the incorporation of tritiated thymidine in tumor cells incubated with DNase I. Programmed cell death is a very active metabolic process. Various authors [30,31] have reported a slow incorporation of radionuclide in cells where apoptosis was induced; those results are similar to ours. Interestingly, slow radionuclide incorporation has also been reported in cells undergoing autoschizis [32].

The advantages of using DNase to inhibit the proliferation of cancer cells are beginning to emerge. Taper et al. have shown that the reactivation of DNase activity provoked by a combined treatment with vitamin C/K_3 in human prostate tumors induced tumor cell death [5]. Similarly, it has recently been shown that the nucleoprotein complex involving an insulator element which corresponds to the DNase I hypersensitivity site upstream of the c-MYC gene in HeLa cells must be reassembled *de novo* with each new cell generation [33], thus reinforcing the importance of considering DNase as an option in cancer treatment. All the above results reinforce the importance of evaluating the use of DNase in other cancer models.

Conclusions

Our study shows the high sensitivity of tumor cells to the presence of DNase I, mainly in cells of tumor origin with a high rate of proliferation. Our results also emphasize the relevance of evaluating the advantages of this nuclease in the control of cancer.

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