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Guanosine Induces Necrosis of Cultured Aortic Endothelial Cells

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We have observed that treatment of cultured bovine aortic endothelial (BAE) cells with guanosine can inhibit the proliferation and viability of the cells. The addition of 500 μmol/L guanosine to the medium resulted in approximately 90% inhibition of cell proliferation. It also changed the morphology of BAE cells from having a small cobblestone-like appearance to a giant pancake-like morphology. At a concentration range of 1 to 2.5 mmol/L, guanosine inhibited the viability of quiescent BAE cells. Incubation of the cells with 2 mmol/L guanosine for 24 hours maximally induced the loss of cell viability by approximately 80%. We also compared the effects of different nucleosides on the proliferation and viability of BAE cells and found that at appropriate concentration ranges, only guanosine was able to inhibit the proliferation and viability of the cells. To assess the mechanism that mediates the cytotoxicity of guanosine, we analyzed the degradation pattern of DNA in guanosine-treated cells and found that random DNA degradation occurred in the cells. Thus, we suggest that treatment of BAE cells with guanosine induced cell necrosis. (Am J Pathol 1994, 145:423–427)

Cell death is a normal and important physiological regulatory process in the development of multicellular organisms. However, unscheduled or uncontrollable cell death seems to contribute to the development of diseases such as Alzheimer and Parkinson diseases, which involve neuron death,[1] and T cell death in AIDS.[2] Therefore, the identification of potential risk factors that induce cell death in various tissues should help us understand the mechanisms by which cell death occurs and lead to effective strategies for the prevention and onset of cell death-related diseases.

The mechanisms by which cell death occurs have been classified into two basic pathways, apoptosis and necrosis.[3–6] Apoptosis is cell specific and genetically programmed.[3,4] The induction of apoptosis has been shown to require the expression of certain genes in cells.[3,4] During the process of apoptosis, the cell body shrinks, chromatin condenses, and DNA degradation is induced by endonucleases.[3,4] This type of DNA degradation can be visualized by resolving degraded DNA through agarose gel electrophoresis. The migrating pattern of the degraded DNA in apoptotic cells is characteristically a 150 to 200-bp ladder because the degradation of DNA in apoptotic cells occurs in the internucleosome regions.[3,4] During this process, the membrane structures remain intact.[3,4] In contrast, in the process of cell necrosis, the cell body swells and severe damage occurs in the plasma membrane, organelle membranes, and nuclear membrane.[5,6] In addition, when the degraded DNA is analyzed in agarose gel by electrophoresis, it displays a smear pattern indicating that DNA degradation occurred randomly.[5,6]

In the case of coronary diseases, the death of endothelial cells lining the lumen of the blood vessels has been linked to the initiation and progression of such diseases as thrombosis and arteriosclerosis.[7–9] In this report, we present data to show that treatment of cultured bovine aortic endothelial (BAE) cells with guanosine can induce cell death via a necrotic mechanism.

Materials and Methods

Falcon brand tissue culture flasks, dishes, and plates were purchased from Fisher Scientific Inc. (Springfield, NJ). DME medium with 4.5 g/L glucose, fetal bovine serum (FBS), phorbol 12,13-dibutyrate (PDBu), 8-Br-cAMP, and nucleosides were purchased from Sigma Chemical Co. (St. Louis, MO). Genistein was purchased from ICN Biochemical, Inc.

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(Costa Meca, CA). Recombinant human tumor necrosis factor-α (TNF-α) was purchased from GIBCO-BRL, Inc. (Grand Island, NY). H-7 and H-8 compounds were purchased from Seikagaku America, Inc. (Rockville, MD). The DNase-free RNase was purchased from Boehringer Mannheim Co. (Indianapolis, IN).

Cell Culture

A strain of primary BAE cell culture was provided by Dr. Robert Auerbach (Zoology Department, University of Wisconsin, Madison, WI). These cells were cultured in the DME medium supplemented with 10% FBS. The medium was also supplemented with 100 U/ml penicillin and 50 U/ml streptomycin. All cultures were kept in a humidified incubator with 5% CO_2 and 95% air at 37 C.

Cell Proliferation Assay

Approximately 3×10^5 cells in 1 ml of the medium were seeded into the wells of 24-well tissue culture plates. The cells were cultured for 24 hours. After this incubation (day 0), the medium was replaced with fresh medium containing agents to be tested and the cells were cultured for another 3 days. At the end, the medium was removed, cells were trypsinized, and the total number of cells per well was determined using a electronic cell counter (Royco model 200TC). Each point was determined in triplicate.

Cell Viability Assay

BAE cells were cultured to confluency in 100-mm tissue culture dishes. The medium was removed and replaced with fresh medium containing various additions as indicated in the text. The cells were incubated for 24 hours. At the end, the medium was removed and the cells were incubated in 2 ml of phosphate-buffered saline (PBS) containing 0.4% trypan blue for 5 minutes at room temperature. The PBS was removed and replaced with dye-free PBS. The stained cells were observed under a light microscope. The percentage of dead cells was determined as the percentage of stained cells from randomly chosen fields. Three fields were observed in each dish and each point was determined by the average of the counts from three dishes.

DNA Degradation Assay

Confluent quiescent monolayers of BAE cells were treated with various reagents as described in the text for 48 hours. At the end, the medium was removed and cells were scraped off the dishes and harvested into 10 ml PBS containing 2 mmol/L EDTA. The cells were pelleted by centrifugation and resuspended in the PBS-EDTA buffer. The cells were resuspended into a single cell suspension. The cell density was determined, and an aliquot of cell suspension containing approximately 5×10^6 cells was centrifuged to pellet the cells. The cells were lysed in 500 µl of a hypotonic buffer (10 mmol/L Tris-HCl, pH 8, 1 mmol/L EDTA, 0.2% Triton X-100). The lysate was incubated on ice for 20 minutes, followed by centrifugation in a micriofuge at 12,500 × *g* for 5 minutes at 4 C. The supernatant was transferred into another microfuge tube and extracted with an equal volume of phenol, followed by chloroform. After this, the salt concentration in the aqueous phase was adjusted to 0.5 M NaCl and the DNA and RNA were precipitated with an equal volume of isopropanol at −85 C overnight. The mixture was thawed on ice. The precipitated DNA and RNA were pelleted then resuspended in 25 µl of TE buffer containing 200 µg/ml DNase-free RNase. This suspension was incubated at 37 C for 30 minutes. After this treatment, the sample was electrophoresed in a 2% agarose gel. The gel was stained with ethidium bromide, destained, and photographed under ultraviolet light.

Results

Inhibition of Cell Proliferation by Guanosine

In recent studies using nucleosides and nucleotides and their effects on BAE cell proliferation, we observed that treatment of BAE cells with low concentrations of guanosine caused inhibition of cell proliferation. Figure 1 demonstrates that treatment of BAE cells with guanosine within a concentration range of 1 mmol/L for 3 days inhibited the proliferation of the cells in a dose-dependent fashion. Based on these data, we estimated that 500 µmol/L of guanosine maximally inhibited the proliferation of BAE cells by approximately 90%. The photographs in Figure 1, a and b were taken at the end of the cell proliferation assay. Figure 1a represents the small cobblestone-like morphology of BAE cells. In contrast, Figure 1b shows that those BAE cells that had been incubated with 500 µmol/L of guanosine for 3 days acquired a giant pancake-like morphology.

The effects of adenosine, cytidine, guanosine, inosine, thymidine, uridine, and xanthine on the proliferation of BAE cells was compared. Figure 2 indicates that at 500-µmol/L concentration, only guanosine significantly inhibited (by approximately 92%) the proliferation of BAE cells. This suggests that at this con-

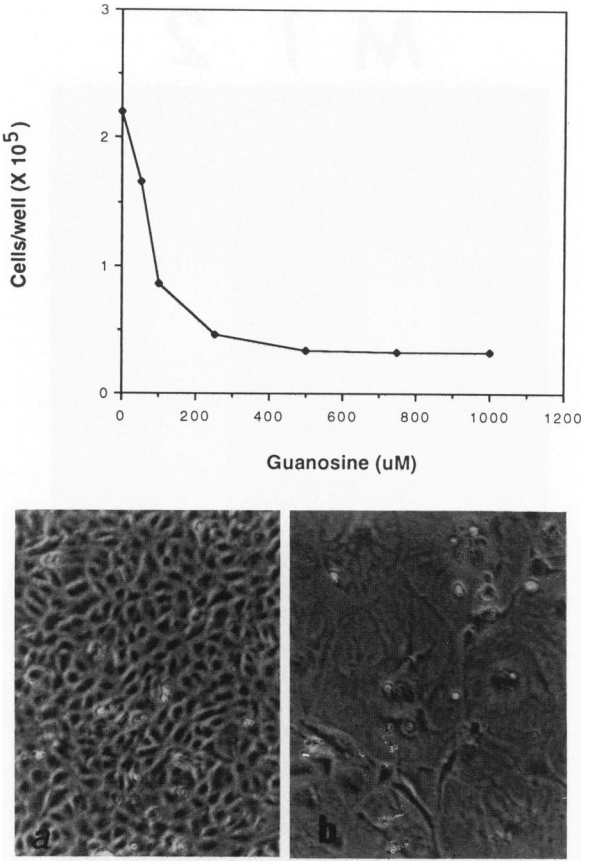

Figure 1. *Inhibition of BAE cell proliferation by guanosine.* **A:** *BAE cells were treated with guanosine at various concentrations for 3 days. Total cell number per well was determined in triplicate at the end of treatment. The morphology of control BAE cells* (**A**) *and guanosine-treated (500 μmol/L guanosine) BAE cells* (**B**) *at the end of the cell proliferation assay were magnified ×100.*

centration range, the proliferation of BAE cells was selectively inhibited by guanosine.

Inhibition of the Viability of BAE Cells by Guanosine

Judging by trypan blue staining of cells, we found that treatment of BAE cells with higher concentrations of guanosine caused the loss of cell viability. Figure 3 indicates that treatment of BAE cells with guanosine within a concentration range of 1 to 2.5 mmol/L for 24 hours inhibited the viability of the BAE cells in a dose-dependent fashion. Guanosine at a concentration of 2 mmol/L maximally inhibited the viability of more than 90% of the BAE cells. Figure 4 shows the comparative effects of various nucleosides on the viability of BAE cells. We found that unlike the effect of guanosine, incubation of BAE cells in the medium containing 2 mmol/L of adenosine, cytidine, inosine, uridine, or xanthine for 24 hours had no effect on the viability of

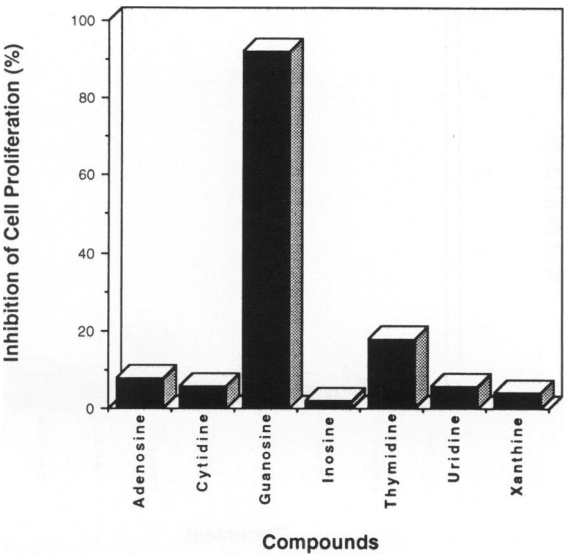

Figure 2. *Comparative effects of different nucleosides on BAE cell proliferation. BAE cells were cultured in the medium containing 500 μmol/L of each individual nucleosides for 3 days. Total cell number per well was determined in triplicate at the end of treatment.*

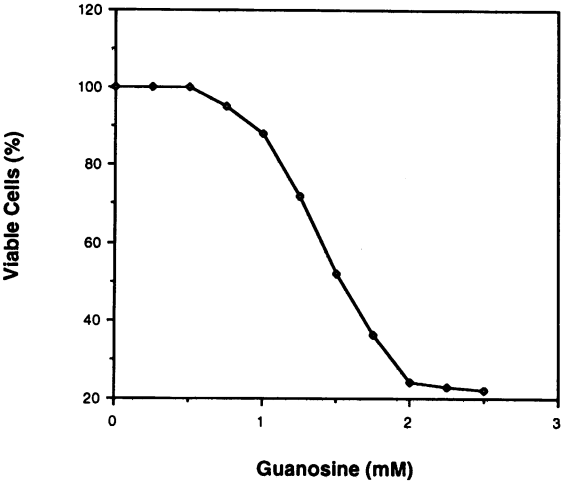

Figure 3. *Guanosine inhibits the viability of the BAE cells in a dose-dependent fashion. Monolayers of BAE cells were treated with guanosine at different concentrations for 24 hours. The cells were stained with 0.4% trypan blue in PBS. The trypan blue-stained cells were taken as dead cells. The percentage of viable cells was determined, as described in the Materials and Methods section.*

the cells. The addition of 2 mmol/L thymidine inhibited the viability of approximately 15 to 20% of BAE cells.

DNA Degradation Occurs in Guanosine-Treated BAE Cells

Figure 5a represents the normal morphology of cultured BAE cells. The photograph in Figure 5b was taken at the end of a 24-hour treatment of BAE cells with 2 mmol/L guanosine. The dark images in Figure 5b were dead cell bodies with severe destruction of the cellular structure. We investigated the stability of

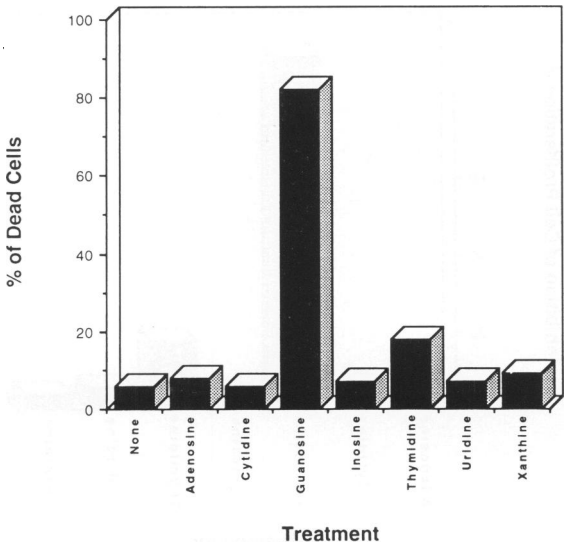

Figure 4. *Comparative effects of various nucleosides on the viability of BAE cells. BAE cells were treated with the listed compounds at a concentration of 2 mmol/L for 24 hours. The viability was determined at the end of this treatment.*

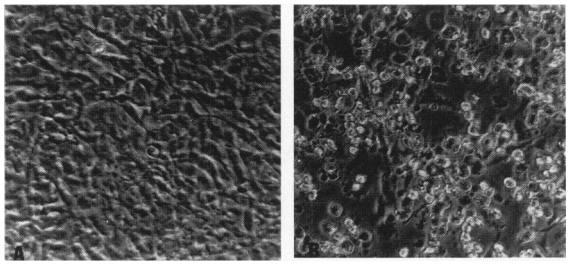

Figure 5. *Guanosine induces cell lysis.* **A:** *The morphology of quiescent BAE cells.* **B:** *The morphology of BAE cells after treatment with 2 mmol/L guanosine for 24 hours. These images are magnified ×200.*

DNA in these cells. Figure 6 (lane 2) indicates that severely random degradation of DNA occurred in BAE cells treated with 2 mmol/L guanosine for 24 hours.

Comparative Effects of Guanosine and Other Agents on Cell Viability

Finally, we compared the effects of guanosine on the viability of BAE cells with several additional non-nucleoside agents. Figure 7 indicates that compared with the effect of guanosine (2 mmol/L), treatment of BAE cells with 1 mmol/L 8-Br-cAMP, 100 nM PDBu, 100 μM genistein, 100 μM H7, 100 μM H8, or 10 ng/ml TNF-α for 24 hours had no effect on the viability of the cells.

Discussion

In this study, we have shown that treatment of cultured BAE cells with guanosine at a low concentration

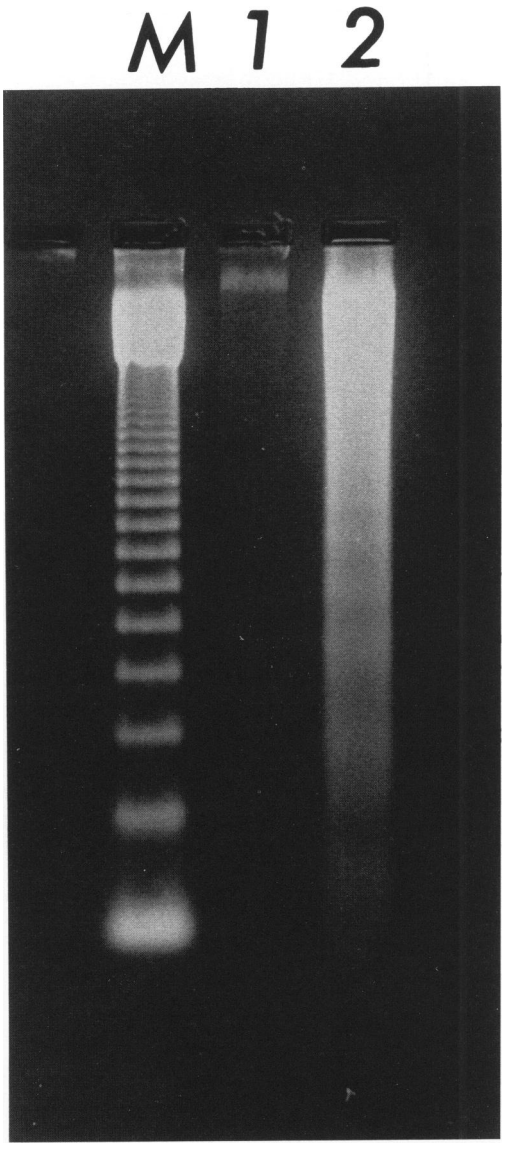

Figure 6. *Guanosine induces random degradation of DNA in BAE cells.* **A:** *Confluent monolayers of BAE cells were treated with 2 mmol/L guanosine for 24 hours. The degraded DNAs were isolated from 5 × 10^6 cells and analyzed in a 2% agarose gel. Lane M contains a 123-bp DNA marker. Lane 1 represents the control cells.*

range (<500 μmol/L) inhibited the proliferation of the cells, whereas at a higher concentration range (1 to 2 mmol/L) it inhibited the viability of the cells. In contrast to guanosine, we found that other nucleosides had little or no inhibitory effect on the proliferation and viability of BAE cells, indicating that the proliferation and viability of BAE cells was selectively inhibited by guanosine. We suggest that the observed inhibitory effect of guanosine on BAE cell proliferation was due to the cytotoxicity of guanosine. Although at a low concentration range (<500 μmol/L) guanosine did not induce immediate BAE cell death, it caused a low

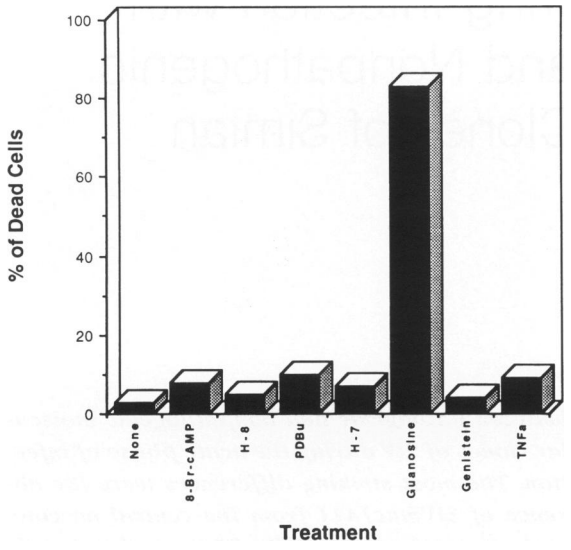

Figure 7. *Comparative effects of various agents on the viability of BAE cells. BAE cells were treated with 1 mmol/L 8-Br-cAMP, 100 μM H-8, 100 nM PDBu, 100 μM H-7, 2 mmol/L guanosine, 100 μM genistein, or 10 ng/ml TNF-α for 24 hours. The viability of the cells was determined at the end of the treatment.*

level of cytotoxicity sufficient to cause inhibition of the BAE cell proliferation.

In addition, we found that in guanosine-treated BAE cells, there was extensive degradation of DNA. Currently, DNA degradation is being used as one measurement for the determination of the efficacy of the cytotoxic drug treatment on cells, particularly as it relates to the process of apoptosis.[10–12] The migrating pattern of the degraded DNA in agarose gel electrophoresis also reveals the mechanisms by which cells die.[3–6] In general, it was observed that in necrotic cells DNA is degraded in a random fashion after the cells lose their membrane structures.[5,6] On the contrary, when cells are dying via apoptosis, the DNA degradation occurs before the destruction of the cellular structures and the degradation of DNA occurs only in the internuclearsome regions.[3,4] Using DNA degradation as a measurement, we found that the DNA in guanosine-treated BAE cells was degraded randomly. This indicates that the death of guanosine-treated BAE cells occurred via a necrotic mechanism.

We have shown that activation of the protein kinase A with 8-Br-cAMP or protein kinase C with PDBu had no effect on the viability of BAE cells. In addition, we found that inhibition of the PKA with H-8, PKC with H-7, or tyrosine kinases with genistein also had no effect on the viability of BAE cells. Therefore, at the biochemical level, guanosine-induced death of BAE cells probably did not involve the pertubation of the activities of these protein kinases.

TNF-α has been known to inhibit the viability of certain types of cancer cells[13] and endothelial cells.[14] In this study we have observed that treatment of BAE cells with 10 ng/ml TNF-α for 24 hours had no observable effect on the viability of the cells. Therefore, it rules out the possibility that TNF-α might be involved in guanosine-induced death of BAE cells. Our data suggest that there might be a physiological regulatory role for guanosine in endothelial cells.

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