Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 258-264

DEOXYRIBONUCLEOSIDE-TRIPHOSPHATE IMBALANCE DEATH: DEOXYADENOSINE-INDUCED dNTP IMBALANCE AND DNA DOUBLE STRAND BREAKS IN MOUSE FM3A CELLS AND THE MECHANISM OF CELL DEATH

Akiko Yoshioka, Shohei Tanaka, Osamu Hiraoka, Yuji Koyama,

Yasuhide Hirota, and Yusuke Wataya

Faculty of Pharmaceutical Sciences, Okayama University,
Tsushima, Okayama 700, Japan

Received May 13, 1987

SUMMARY: The mechanism of deoxyadenosine (dAdo)-induced death of mouse mammary tumor FM3A cells was studied. When the cells were exposed to dAdo at 3 mM, an imbalance of intracellular dNTP pool resulted: dATP concentration was elevated 100-fold and the dGTP concentration was reduced to less than 1% of the control values. The imbalance was followed by breakage of mature DNA. DNA double strand breaks were observed in the dAdo treated cells 12 hr after the administration. We assume that the double strand breaks play an important role in the process of the dAdo-mediated cell death, and that the intracellular dNTP imbalance is the trigger of these events.

An inherited deficiency of adenosine deaminase (ADA) is associated with combined immunodeficiency diseases (1). Deoxyadenosine (dAdo), when administered with ADA inhibitor deoxycoformycin, is cytotoxic to T-lymphocytes (2,3). There is a large amount of dATP in ADA deficient cells (4), and it is believed that the accumulation of dATP inhibits the ribonucleotide reductase and DNA synthesis in dividing cells (2). In non-dividing cells, the large amount of dATP and imbalance of intracellular dNTP pool inhibit the DNA repair (3). Inhibition of DNA synthesis or DNA repair can cause cell death (2,3,5). The question arises why the cells including the dividing and the non-dividing cells die under these stresses. Recently, we proposed a mechanism for the 5-fluorodeoxyuridine (FdUR)-induced cell death on the basis of our observation that DNA double strand breaks take place subsequent to the formation of deoxynucleoside triphosphate (dNTP) imbalance (6). The pattern of the dNTP imbalance in the FdUR treated cells is the accumulation of dATP and depletion of both dGTP and dTTP. Since the accumulation of dATP is also observed for the

0006-291X/87 $1.50

cytotoxic action of dAdo, it is possible that DNA double strand breaks occur in these cells. If that is the case, the dAdo cytotoxicity may be explained in terms of the formation of DNA double strand breaks which should be lethal to the cells.

We have now measured the levels of intracellular dNTP pool, DNA strand breaks and cell viability in dAdo treated FM3A cells and compared the results with those found for the FdUR treated cells. We have found that the dNTP imbalance caused by dAdo is followed by DNA double strand breaks and subsequent cell death.

MATERIALS AND METHODS

Materials. Deoxyadenosine was a product of Yamasa Chemicals (Japan), cycloheximide that of Wako Chemicals (Japan), 1,1,2-trichlorotrifluoroethane that of Aldrich (USA), and tri-n-octylamine that of Tokyo Kasei (Japan). Mouse mammary tumor FM3A cells (wild type F28-7) (7) were generously given by Dr. T. Seno (Saitama Cancer Center Research Institute, Japan).

Cell culture. FM3A cells were maintained in suspension culture at 37°C in a 5% CO_2 atmosphere in plastic bottles (Nunclon, Denmark) containing ES medium (Nissui Seiyaku, Japan) (7) supplemented with 2% heat-inactivated fetal bovine serum (GIBCO, USA). Cell numbers were measured using a micro cell counter CC-108 (Toa Electric, Japan) or using a blood cell counting chamber.

For growth inhibition studies, cells were seeded at a density of about 5 x 10^4 cells/ml, and immediately treated with various concentrations of a reagent. The reagent was present in the medium during the entire period of the culture. Cell numbers were determined 48 hr after the addition of the reagent. EC_{50} value refers to the concentration of reagent necessary to inhibit the increase in cell density at 48 hr by 50% of the control.

Determination of cell viability. Cell viability during treatment with a reagent was determined either by staining with trypan blue or by measuring the colony forming ability. For the latter, cells were collected by centrifugation (100 x g at 37°C for 10 min), washed twice with a fresh medium at 37°C and then resuspended in a small volume of fresh medium. After counting the cell number, the suspension was diluted with the fresh medium to give a final density of 3000 cells/ml. 100 μl of the cell suspension was innoculated onto the surface of an agar medium consisting of ES medium supplemented with 5% fetal bovine serum and 0.5% agar (purified agar, Difco, USA) which was placed in a tissue culture dish (90 x 20 mm, Nunclon). After incubation for 10 days at 37°C and in a 5% CO_2 atmosphere, colonies formed were counted.

Quantitative determination of dNTP. Cells were grown to a density of 2 x 10^5 cells/ml, and then dAdo (3 mM) was added. At specified times, cells (50 ml portions of the suspension) were harvested by centrifugation at 100 x g at 4°C for 10 min. The cells were washed in phosphate buffered saline (PBS) (containing 0.1% glucose) and centrifuged. After the cell pellet was resuspended in PBS, the cell number was counted. The cells were then extracted with trichloroacetic acid and the extract was neutralized with a Freon-Amine solution (0.5 M tri-n-octylamine in 1,1,2-trichlorotrifluoroethane). For quantitative determination of dNTP, the extract was treated with periodate and methylamine to decompose the ribonucleotides, and then the

mixture was subjected to analysis by HPLC. The details are described in our earlier publication (8).

Analysis of DNA strand breaks DNA strand breaks were analyzed by the alkaline and neutral elution methods or by the alkaline sucrose density gradient sedimentation. For the elution analysis, cells were grown for 24 hr in ES medium containing 1.2 μM [2-^{14}C]-thymidine (55.2 Ci/mol, New England Nuclear). Cultures were subsequently incubated in the absence of label to chase all the radioactivity into the high molecular weight DNA. The cells (2 x 10^5 cells/ml) bearing [^{14}C]-DNA were incubated with dAdo (3 mM) in ES medium containing serum at 37°C in 5% CO_2 environment. At the specified time, cells (5 x 10^5 cells) were carefully rinsed with 10 ml of PBS and loaded onto Swinnex (Millipore) funnels as described by Kohn et al. (9). The cells were lysed on the filter with a mixture of sodium dodecyl sulfate and proteinase K at 25°C for 1 hr. The filter was then eluted with a buffer at pH 12.0 for the alkaline elution, or at pH 9.6 for the neutral elution. This procedure was essentially identical with that described by Kohn and coworkers. (10,11).

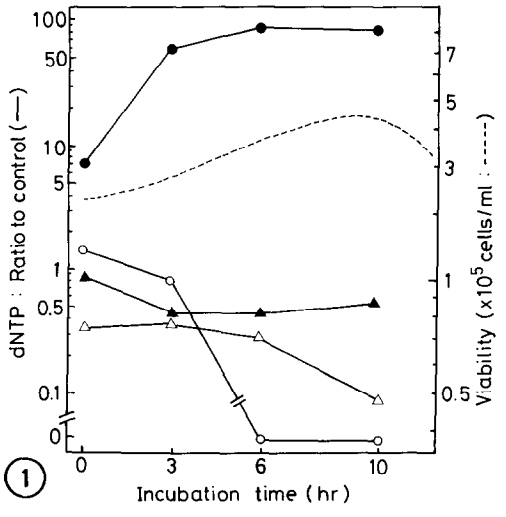
In the alkaline sucrose density gradient sedimentation, cells were labeled for 24 hr with 4.3 nM [methyl-^{3}H]-thymidine (47 Ci/mol, New England Nuclear), and chased in fresh ES medium for 24 hr. The cell density was then adjusted to about 2 x 10^5 cells/ml, and dAdo was added. At a desired time, cells were collected and washed with PBS. The washed cells were resuspended in cold PBS at a density of about 1 x 10^6 cells/ml. The sedimentation analysis was performed as described by Rawles and Collings (12).

RESULTS

The growth inhibitory activity of dAdo and some other nucleosides was examined. The EC_{50} value for dAdo on F28-7 was 1.6 x 10^{-4} M. The EC_{50} for deoxyguanosine was 2.5 x 10^{-5} M and that for deoxythymidine was 1.6 x 10^{-4} M. No inhibition of cell growth was observed for deoxycytidine at its concentration of 2 x 10^{-3} M.

In terms of the colony forming ability, the cell death began at 8 hr of exposure to 3 mM dAdo. At 24 hr, colony formation at only 2 % of control was observed. The 3 mM dAdo-induced cell death was prevented by addition of cycloheximide at 2 μg/ml (data not shown).

Fig. 1 shows the time-dependent change in the cellular dNTP-pool on treatment with 3 mM dAdo. With this concentration of dAdo, the cell growth was inhibited to 10% of the control. This treatment resulted in significant changes in the intracellular dNTP pools. Within 6 hr during the treatment, dATP became about 100-fold the amount at zero-time, and the dGTP-pool size became less than the lower limit of measurement. By the 6 hr treatment, no significant changes took place in the dTTP and dCTP contents. Intracellular ribonucleoside-triphosphate (rNTP) pools were not changed by the dAdo treatment.



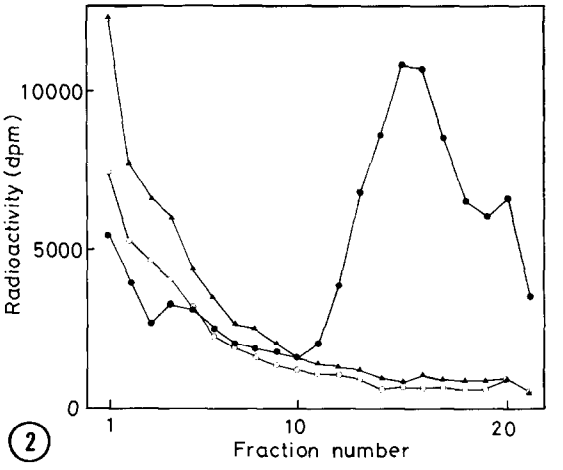


Figure 1. dNTP pool changes and cell viability in FM3A (F28-7) cells treated with 3 mM dAdo. F28-7 cells at a density of 2×10^5 cells/ml were treated with 3 mM dAdo. The total volume used was 1000 ml. At the indicated times, aliquots of 50 ml were removed and the dNTP pools were measured as described in Methods. Cell viability was determined by staining with trypan blue. ● : dATP, ○ : dGTP, △ : dCTP, and ▲ : dTTP

Figure 2. Determination of DNA strand breakage during dAdo treatment by alkaline sucrose density gradient centrifugation. F28-7 cells, in which the mature DNA strands were labeled, were treated with 3 mM dAdo for 0 hr (○), 3 hr (▲), and 24 hr (●). 20 μl of the cell suspension was layered on the top of 200 μl of a lysing solution (0.4 M NaOH, 0.9 M NaCl, 10 mM Na_2EDTA and 1% Salcosyl). Cells were lysed in the dark for 1 hr at room temperature and centrifuged in a Hitachi RPS 50-2 rotor. After centrifugation, fractions were collected from the bottom of the tube. The collected fractions were neutralized with acetic acid and counted for radioactivity in Triton X-100 liquid scintillator. Sedimentation was from the right to the left.

The treatment with dAdo induced DNA strand breaks in FM3A cells, as evidenced by analysis of the cellular DNA with sedimentation in alkaline sucrose density gradient (Fig. 2). With the 3 mM dAdo treatment for 3 hr, the DNA sedimented to the bottom of the gradient as in the control; however, the treatment for 24 hr resulted in fragmentation of the DNA into smaller pieces. The sedimentation coefficient of the major DNA fragment formed (fractions 15 and 16) was about 65S.

DNA single- and double-strand breaks, as measured by the alkaline and neutral filter elution methods, were detected at 15 hr after the treatment with 3 mM dAdo (Fig. 3). The level of the single-strand breaks was approximately 150-rad γ-ray equivalent. In contrast, the DNA double-strand breaks were equivalent to those inducible by the γ-irradiation at 2450 rads. This

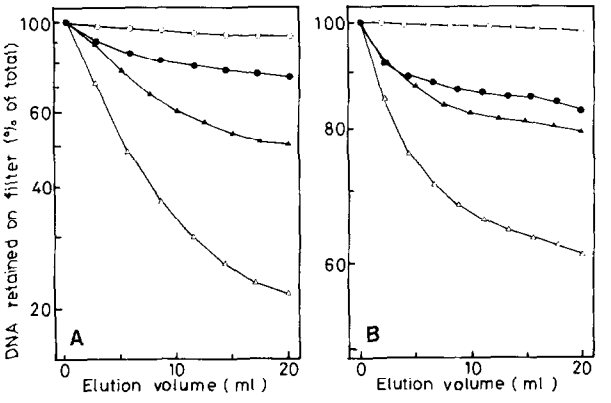
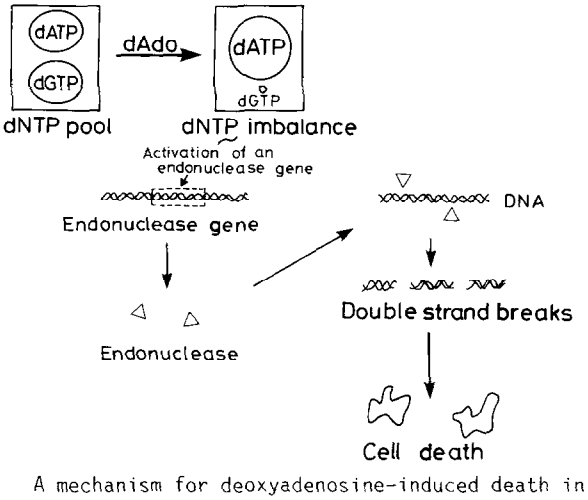


Figure 3. Alkaline and neutral elution patterns for dAdo-treated cells. F28-7 cells prelabeled with [^{14}C]-thymidine were incubated with 3 mM dAdo for 15 hr (●). A and B are alkaline and neutral elution patterns, respectively. For comparison, results of experiments in which exponentially growing cells were irradiated with 300 rads (▲) or 600 rads (△) of γ-ray are shown in A, and those in which the cells were irradiated with 3000 rads (▲) or 6000 rads (△) in B.

suggests that the dAdo-induced double strand breaks were not a result of an accumulation of single-strand breaks, but rather were formed by direct double-strand cuts.

DISCUSSION

Our hypothesis for the mechanism of dAdo-induced cell death is shown in Scheme 1. The dNTP pool imbalance induced by dAdo would be a signal for activating a gene for an endonuclease. The gene product thus induced would



Scheme 1. A mechanism for deoxyadenosine-induced death in FM3A cells.

then attack the DNA to cause double strand breaks. Since an accumulation of DNA double strand breaks should be lethal for living cells, cell death results.

In previous experiments, we showed that treatment of FM3A cells with FdUR caused a dNTP-imbalance, which was followed by DNA double strand breaks and subsequent cell death (6). The pattern of dNTP imbalance induced by FdUR is accumulation of dATP and depletion of dGTP and dTTP. It appears, therefore, that a specific combination of dATP increase and dGTP decrease may be a trigger for the cell death.

It is known that cycloheximide, an inhibitor for protein synthesis, inhibits both the thymine-less death (13) and the FdUR-induced cell death (6) in FM3A cells. We have examined its effect on the dAdo-induced cell death, finding that cycloheximide again prevents the death. This suggests that the dAdo-induced cell death requires protein biosynthesis.

It is known that a large amount of dATP and dADP accumulates in the erythrocytes of adenosine deaminase (ADA) deficient patients (4). The same accumulation occurs in T-lymphocytes on treatment with dAdo and deoxycoformycin (2,3). It is also known that a simultaneous administration of dAdo and deoxycoformycin causes DNA single strand breaks in mature human T-lymphocytes (14). The same combination also gives rise to the inhibition of DNA repair in resting human lymphocytes (3,5). On the basis of our results shown in this paper, these cellular deficits may be explained as resulting from intracellular accumulation of dATP. The large amount of intracellular dATP, which causes the imbalance of dNTP pool, can be a trigger of the lymphocyte death leading to the ADA deficient immunodeficiency.

ACKNOWLEDGMENTS

Professor H. Hayatsu of the Okayama University is gratefully ackowledged for his valuable discussions. This work was supported in part by grants for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Giblett, E. R., Anderson, J. E., Cohen, F., Pollara, B., and Meuwissen, H. J. (1972) Lancet 2, 1067-1069.

2. Ullman, B., Gudas, L. J., Cohen, A., and Martin, D. W., Jr. (1978) Cell 14, 365-376.
3. Cohen, A., and Thompson, E. (1986) Cancer Res. 46, 1585-1588.
4. Coleman, M. S., Donofrio, J., Hutton, J. J., Hahn, L., Daoud, A., Lampkin, B., and Dyminski, J. (1978) J. Biol. Chem. 253, 1619-1626.
5. Seto, S., Carrera, C. J., Wasson, D. B., and Carson, D. A. (1986) J. Immunol. 136, 2839-2843.
6. Yoshioka, A., Tanaka, S., Hiraoka, O., Koyama, Y., Hirota, Y., Ayusawa, D., Seno, T., Garrett, C., and Wataya, Y. J. Biol. Chem. in press.
7. Ayusawa, D., Koyama, H., Iwata, K., and Seno, T. (1981) Somat. Cell. Genet. 7, 523-534.
8. Tanaka, K., Yoshioka, A., Tanaka, S., and Wataya, Y. (1984) Anal. Biochem. 139, 35-41.
9. Kohn, K. W., Ewig, R. A. G., Erickson, L. C., and Zwelling, L. A. (1981) in DNA Repair, A Laboratory Manual of Research Procedures (Friedberg, E. C., and Hanawalt, P. C., eds.) Vol. 1, Part B, pp 379-418, Marcel Dekker Inc., NY.
10. Kohn, K. W., Erickson, L. C., Ewig, R. A. G., and Friedman, C. A. (1976) Biochemistry 15, 4629-4637.
11. Bradley, M. O., and Kohn, K. W. (1979) Nucleic Acids Res. 7, 793-804.
12. Rawles, J. W., Jr., and Collings, J. M. (1977) J. Biol. Chem. 252, 4762-4766.
13. Ayusawa, D., Shimizu, K., Koyama, H., Takeishi, K., and Seno, T. (1983) J. Biol. Chem. 258, 12448-12454.
14. Brox, L., Ng, A., Pollock, E., and Belch, A. (1984) Cancer Res. 44, 934-937.