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MITOCHONDRIAL DAMAGE FOLLOWING EXPOSURE OF ORGANOTYPIC CULTURES OF HUMAN MALIGNANT GLIOMAS TO 2-CHLORO- AND 2-BROMO- 2'-DEOXYADENOSINE

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The effects of 2-chloro-2'-deoxyadenosine (cladribine, 2-CdA) and a closely related compound 2-bromo-2'-deoxyadenosine (2-BdA) on organotypic cultures of human malignant gliomas were studied with the use of electron microscopy. The cultures grown from surgical biopsies included six glioblastomas, three anaplastic astrocytomas and low-grade fibrillary astrocytoma. After 6 to 10 days of the *in vitro* growth the cultures were exposed to 0.3-10 μM 2-CdA or 2-BdA for 1 to 10 days. Mitochondrial swelling and disappearance of cristae following exposure to the tested substances were observed, but only in highly anaplastic (low-differentiated) tumor cells. The mitochondrial toxicity was dose- and time-dependent, and no difference was found between the effect of 2-CdA and 2-BdA.

Key words: *glial tumors, cladribine (2-CdA), 2-bromo-2'-deoxyadenosine, organotypic cultures, mitochondrial toxicity*

When seven patients with untreatable astrocytomas were treated with a new antileukemic and immunosuppressive nucleoside 2-chloro-2'-deoxyadenosine (cladribine, 2-CdA) by Saven et al. (1993), two partial remission lasting several months were noted. This report stimulated us to study the activity of this drug against human malignant gliomas *in vitro*.

Our previous studies showed that tissue extracts of some human gliomas demonstrate markedly higher 2-CdA-phosphorylating activity than extracts of nonmalignant brain tissues (Grieb et al. 1993), and that some gliomas grown in dissociated cultures upon exposure to 2-CdA develop a dose-dependent block of the S-phase of the cell cycle at submicromolar concentrations, and apoptotic fragmentation of nuclear DNA may also occur (Grieb et al. 1995). In the present study we were looking, with the use of electron microscopy, for possible ultrastructural changes in glioma cells in organotypic culture following exposure to 2-CdA, and to a closely related compound, 2-bromo-2'-deoxyadenosine, (2-BdA). The organotypic culture technique was chosen because it faithfully reproduces the three dimensional patterns of the *in vivo* histologic organization and differentiation of tumors, while suppressing the proliferation and outgrowth of the less differentiated cells (Rubinstein et al. 1973). The results should, therefore, be complementary to those obtained with dissociated cultures which favor survival of clonogenic population of tumor cells.

Material and methods

2-CdA and 2-BdA were synthesized by dr. Z. Kazimierczuk, Department of Biophysics, University of Warsaw. Tissue culture media and reagents were purchased from Sigma Chemical Co.

Biopsies from 10 patients with intracranial glial tumors were dissected out at surgery and immediately transferred under sterile conditions to the nutrient culture medium. Macroscopically representative tissue fragments from the tumor were selected for tissue culture preparation. The fragments were cut into 0.5 – 0.7 mm thin slices and placed on collagen-coated glass coverslips with two drops of nutrient medium, sealed in Maximow double assemblies and incubated in a lying drop position at 36.5°C in a standard tissue culture incubator. The nutrient medium consisted of 10% fetal calf serum and 90% RPMI supplemented with glucose to a final concentration of 600 mg%, and with antibiotics. The medium was renewed twice weekly. The experiments were started 6–10 days after initiation of cultures. The selected cultures were exposed for the 24 hours, 3, 7, or 10 days to a medium supplemented with 2-CdA or 2-BdA in the final concentration of 0.3, 1, 3 and 10 μM. Control cultures were grown under standard conditions.

The experimental and control cultures were inspected in a living state under phase-contrast

microscopy, and at prescheduled times they were fixed for electron microscopy. Briefly, the cultures were treated with 2% cold glutaraldehyde for 1 h, washed in cacodylate buffer pH 7.2–7.4, postfixed in 2% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Ultrathin serial sections of material were counterstained with lead citrate and uranyl acetate and examined under a JEM 1500 XB electron microscope.

The remaining tumor tissue was fixed in formol and prepared for conventional light microscopy staining in order to establish the histology of tumors. The tumors were classified according to the criteria of the WHO classification of tumors of the central nervous system (1993). The biopsies included six glioblastomas, three anaplastic astrocytomas and one low-grade fibrillary astrocytoma.

Results

The light microscopy observation of gliomas *in vitro* revealed the initial stage of explants' proliferation within 24 hours. Even in a well-differentiated astrocytoma first delicate processes could be seen around the explant after 1–2 days. During the following days *in vitro*, the neoplastic cells underwent migration from the explant to the outgrowth zone without discrimination of shape (Fig. 1). In a low-differentiated malignant glioma, the highly anaplastic, multinucleated cells were visible in a considerable distance from the explant (Fig. 2). Tumor cell polymorphism characteristic of the glioblastoma *in situ* remained well preserved.

The cultures exposed to 2-CdA or 2-BdA maintained the same histological pattern of the explant and outgrowth zone, as the control cultures (Fig. 3). The migration of cells to the outgrowth zone occurred independently on the concentration of the tested substances and duration of exposure. The morphology of anaplastic cells was similar to that documented in sister control cultures (Fig. 4).

The ultrastructural analysis of the control cultures of malignant glioma (anaplastic astrocytoma – grade III, glioblastoma – grade IV) exhibited polymorphic population of neoplastic cells composed of both well-differentiated and highly anaplastic glial cells. Many cells exhibited features of fibrous astrocytes with more or less abundant cytoplasm containing numerous filaments. The cytoplasm of some tumor cells was almost entirely filled with compact bundles of gliofilaments. Mitochondria, short channels of granular endoplasmic reticulum, lysosomes and dense bodies were scattered in the cytoplasm among the filaments. The cells were often multinucleated or displayed lobulated nuclei with deep invaginations of nuclear membrane (Fig. 5). Low-differentiated neoplastic cells were characterized by small nuclei surrounded sparsely by cytoplasm containing a small number of organellae and a few gliofilaments.

Electron-microscopic examination of the cultures exposed to 2-CdA or 2-BdA revealed abnormalities, which were concentration – and time-dependent, and limited to mitochondria of anaplastic tumor cells. The drugs tested at 0.3 and 3 μM final concentration did not induce distinct mitochondrial toxicity until 10 days of exposure. Both the well-differentiated neoplastic astrocytes and highly anaplastic tumor cells displayed intact ultrastructural features, identical with those observed in control cultures. Exposure to 2-CdA or 2-BdA at 10 μM concentration induced in anaplastic cells characteristic mitochondrial changes. In the low-grade fibrillary astrocytoma, the majority of well-differentiated cells exhibiting the features of fibrillary astrocytes retained intact mitochondria

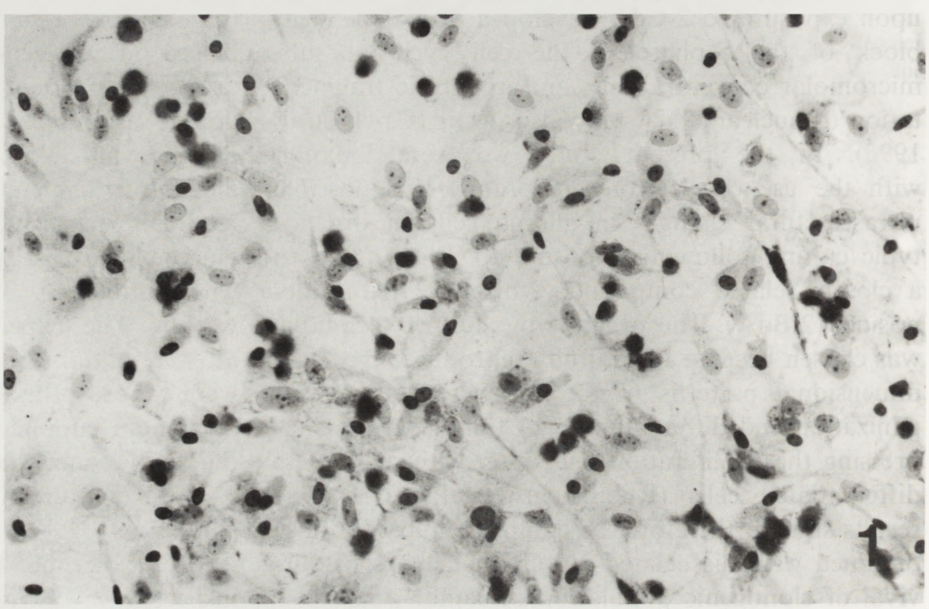

Fig. 1. Control culture of *astrocytoma anaplasticum*, 18 DIV. Widespread outgrowth zone composed of neoplastic cells with fine cellular processes. × 100

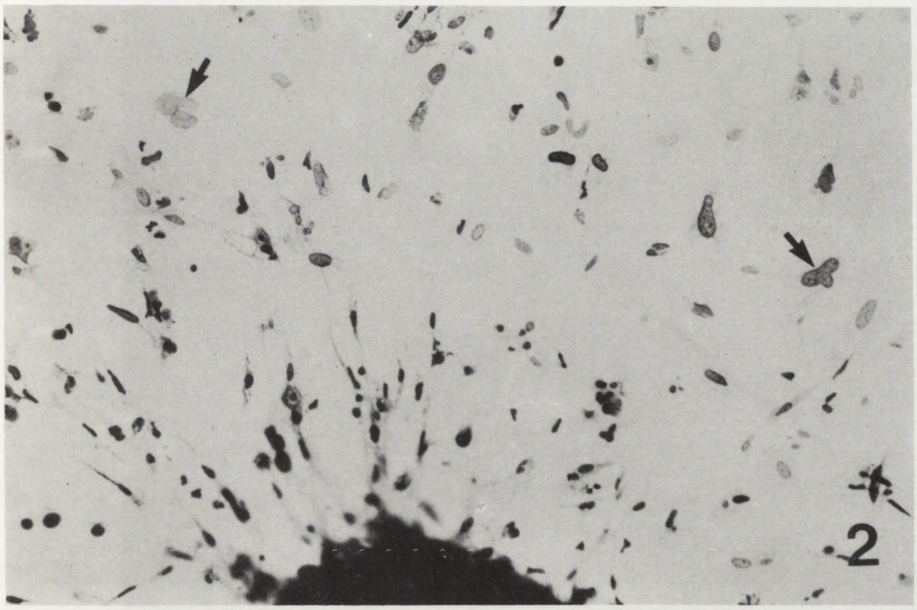

Fig. 2. Control cultures of glioblastoma, 20 DIV. Polymorphic, multinucleated tumor cells (arrows) in the outgrowth zone. ×200

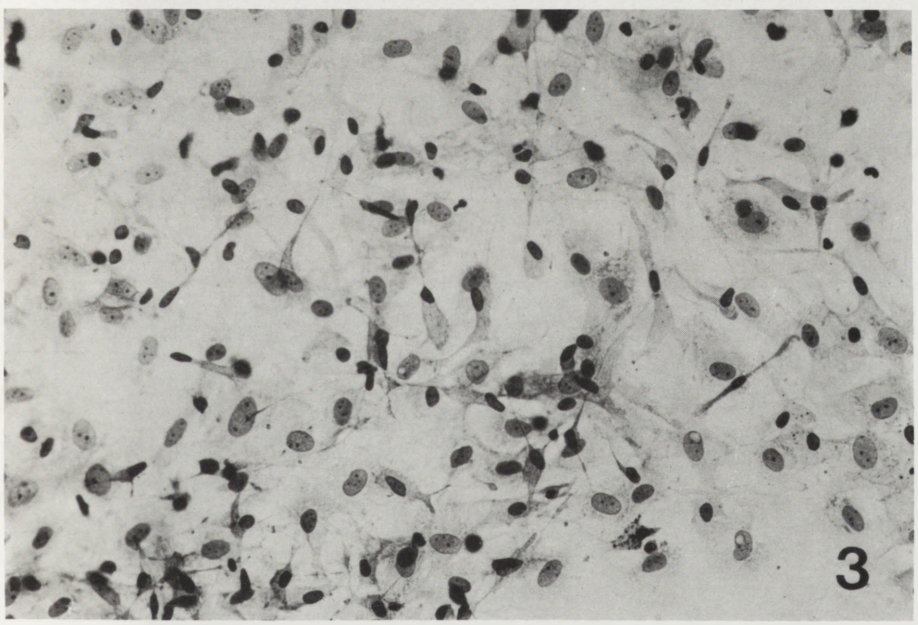

Fig. 3. *Astrocytoma anaplasticum* 15 DIV, 7 days of exposure to 10 μM 2-CdA. Well preserved neoplastic cells in the explants and outgrowth zone. ×100

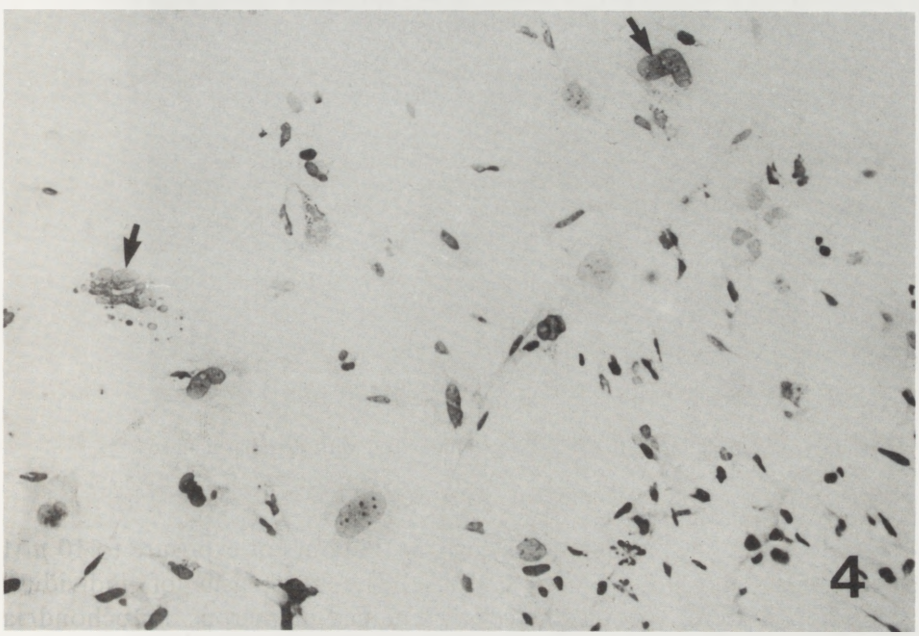

Fig. 4. Glioblastoma 18 DIV, 10 days of exposure to 10 μM 2-CdA. Anaplastic, multinucleated tumor cells (arrows) in the outgrowth zone. ×200

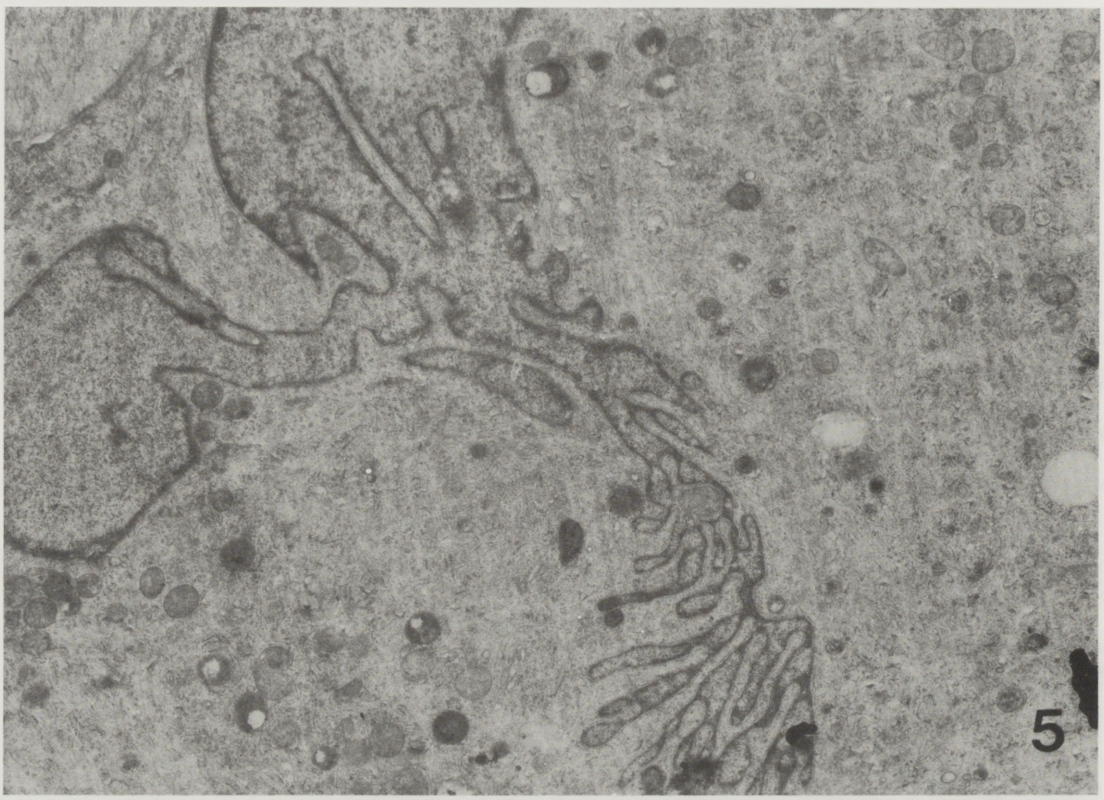

Fig. 5. Control culture of glioblastoma, 18 DIV. Anaplastic tumor cell exhibiting highly lobulated nucleus and abundant cytoplasm packed with glial filaments. ×8000

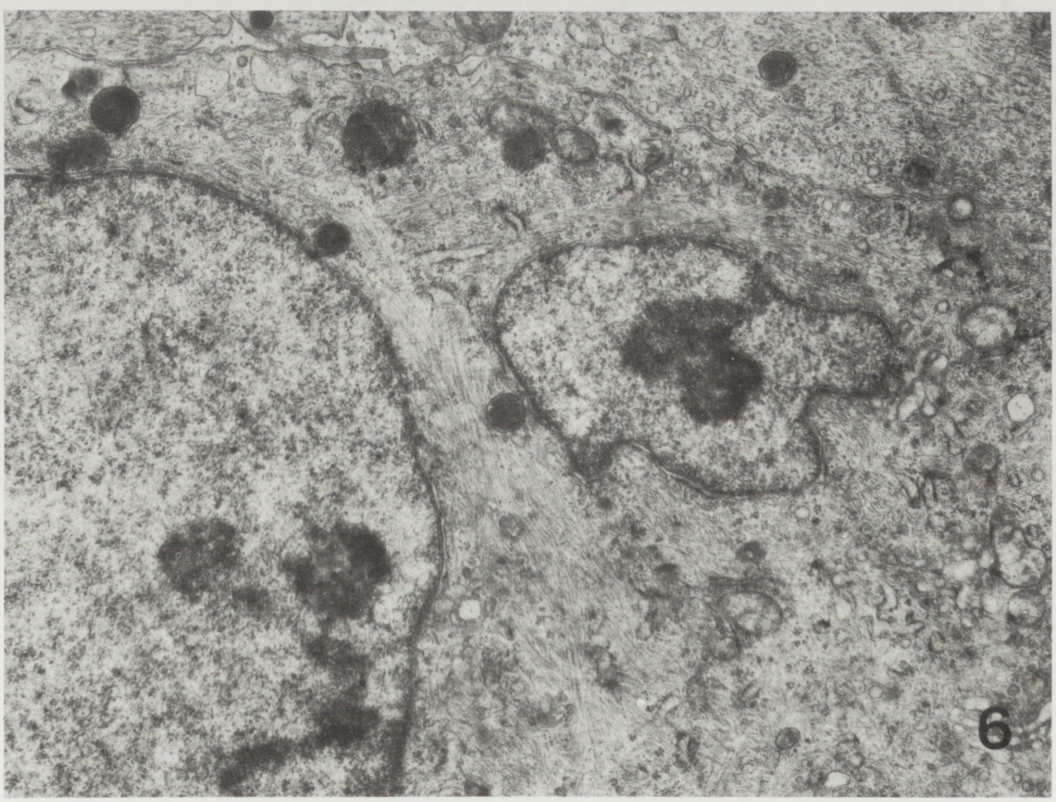

Fig. 6. *Astrocytoma fibrillare*, 10 days of exposure to 10 μM 2-BdA. Tumor cell with cytoplasm filled with gliofilaments. ×15000

even at long exposure times (Fig. 6). However, in malignant gliomas (anaplastic astrocytoma, glioblastoma) the mitochondrial alterations were evidenced as early as 24 hours of exposure to 10 μM 2-CdA or 2-BdA. The cytoplasm of individual neoplastic cells contained numerous mitochondria

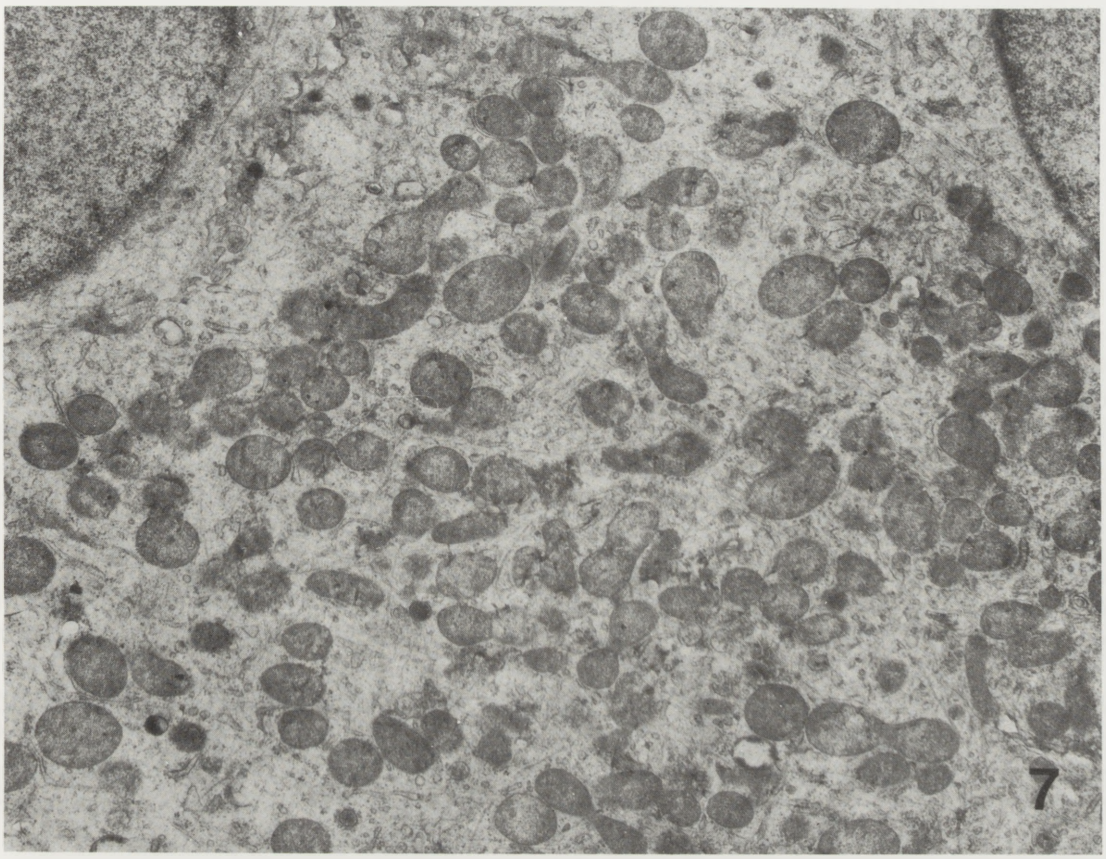

Fig. 7. *Astrocytoma anaplasticum* 10 DIV, 24 hours of exposure to 10 μM 2-CdA. Accumulation of mitochondria lacking internal pattern of cristae. $\times$ 12 000

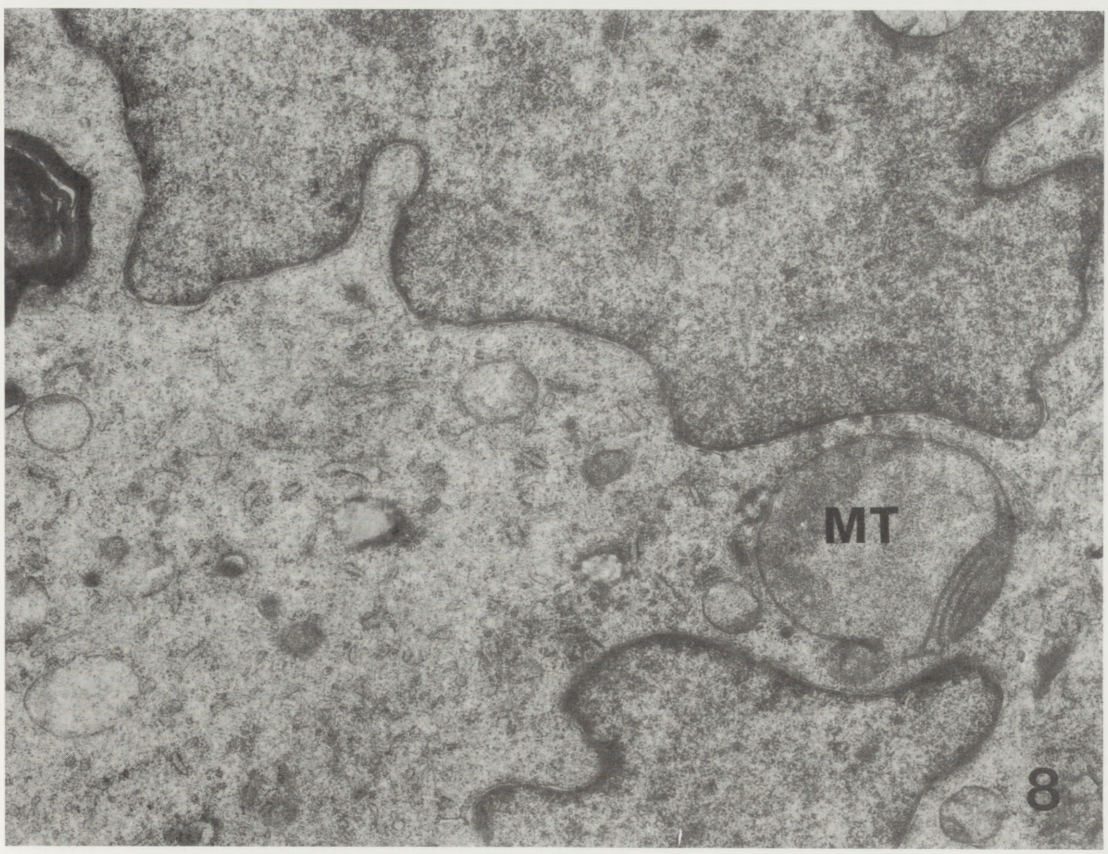

Fig. 8. Glioblastoma 9 DIV, 24 hours of exposure to 7 μM 2-BdA. Severely swollen, enormous mitochondrium (MT) with remnants of cristae. $\times$ 15 000

with dark matrix (Fig. 7). The severely swollen, enormous mitochondria, containing the remnants of cristae adhering to the inner membrane, could be seen occasionally (Fig. 8). After 3 and 7 days of exposure a great majority of anaplastic tumor cells exhibited various degree of mitochondrial changes. Both slightly swollen and completely damaged mitochondria lacking internal cristae were present in the cytoplasm of some cells (Fig. 9). However, the most advanced mitochondrial changes were evidenced after 7–10 days of exposure to 2-CdA or 2-BdA at 10 μM. At this time the majority of anaplastic cells, with deeply invaginated or lobulated nuclei, exhibited abundance of damaged mitochondria. The cytoplasm of these anaplastic cells was frequently packed with enlarged mitochondria, lacking their cristae (Fig. 10). However, the swollen, enlarged mitochondria, despite progressive disruption of internal structure, retained intact double membranes. Moreover, the nucleus remained usually unaffected. The abnormalities in chromatin distribution consisting of clumping beneath the nuclear membrane could be seen only sporadically, accompanied by advanced mitochondrial changes, presence of numerous dark bodies, lysosomes and vacuoles. (Fig. 11).

Discussion

2-CdA proves particularly useful in the treatment of indolent lymphoid malignancies such as hairy cell leukemia, B-cell chronic lymphocytic leukemia (B-CLL) and low-grade non-Hodgkin lymphoma, because it is toxic towards both proliferating and resting lymphoid cells (Beutler et al. 1991; Beutler 1992). In these slowly progressing tumors the fraction of proliferating cells is very low and the increase in tumor load occurs mainly because the disposal of non-dividing cells is inefficient. In B-CLL , for example, clinical progression of the disease is associated, somewhat paradoxically, with a loss of clonogeneic potential of the malignant cells (Dadmarz et al. 1990).

In gliomas (including fast-growing *glioblastoma multiforme*, GBM) the fraction of proliferating cells is also small. The incidence distributed throughout the tumor tissue does not exceed 1 per 1,000 viable cells. Stathmokinetic analysis performed with glioma patients by Hoshino et al. (1979) revealed that *in vivo* less than 5 cells per thousand enter cell cycle per hour, and in some anaplastic astrocytomas this number was below the detection limit (<0.1).

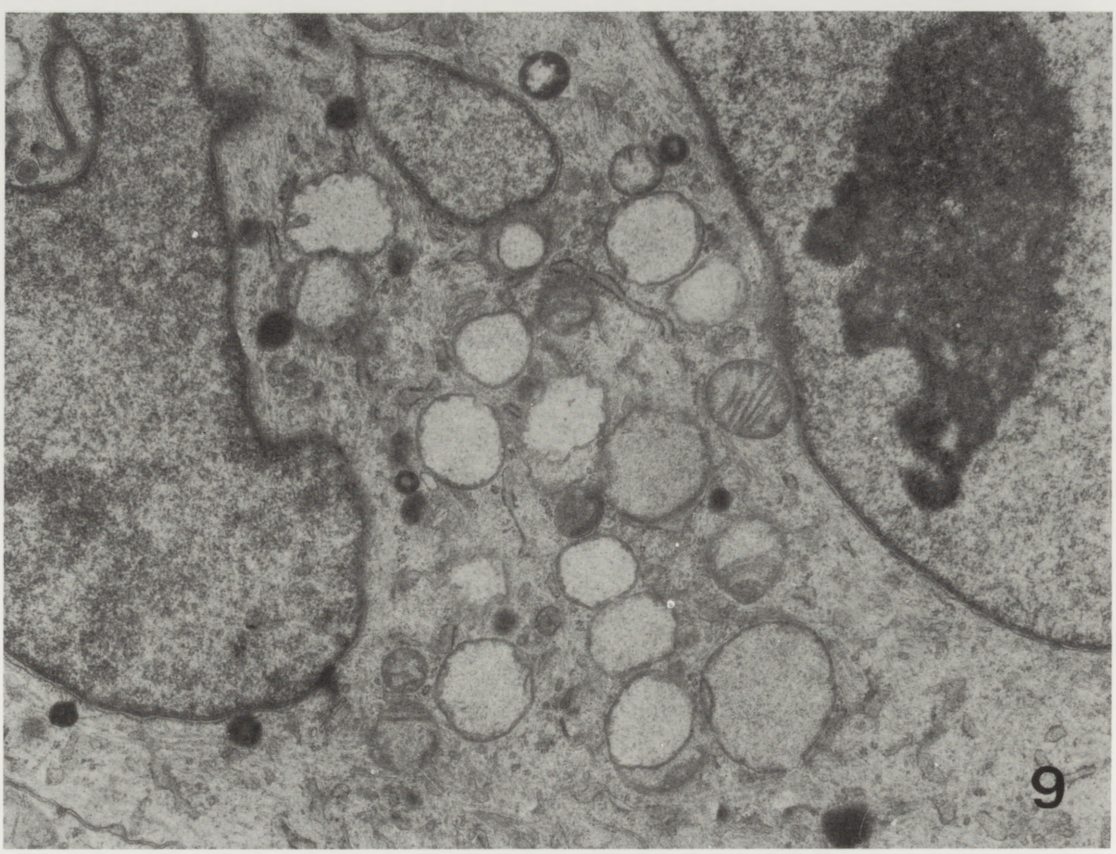

Fig. 9. Glioblastoma 12 DIV, 3 days of exposure to 10 μM 2-CdA. Numerous more or less damaged mitochondria in the cytoplasm of multinucleated neoplastic cell. $\times 15\,000$

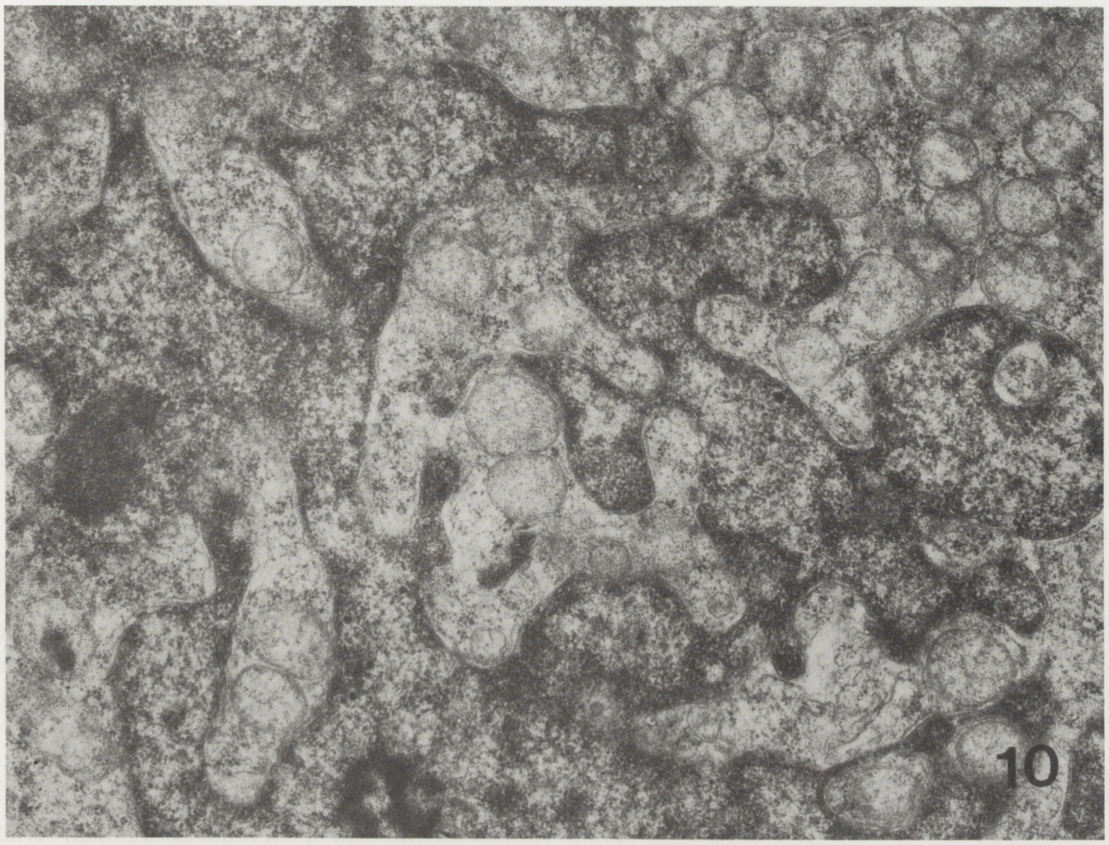

Fig. 10. Glioblastoma 16 DIV, 7 days of exposure to 10 μM 2-CdA. Severely swollen mitochondria in the perinuclear cytoplasm of anaplastic tumor cell. $\times$ 20 000

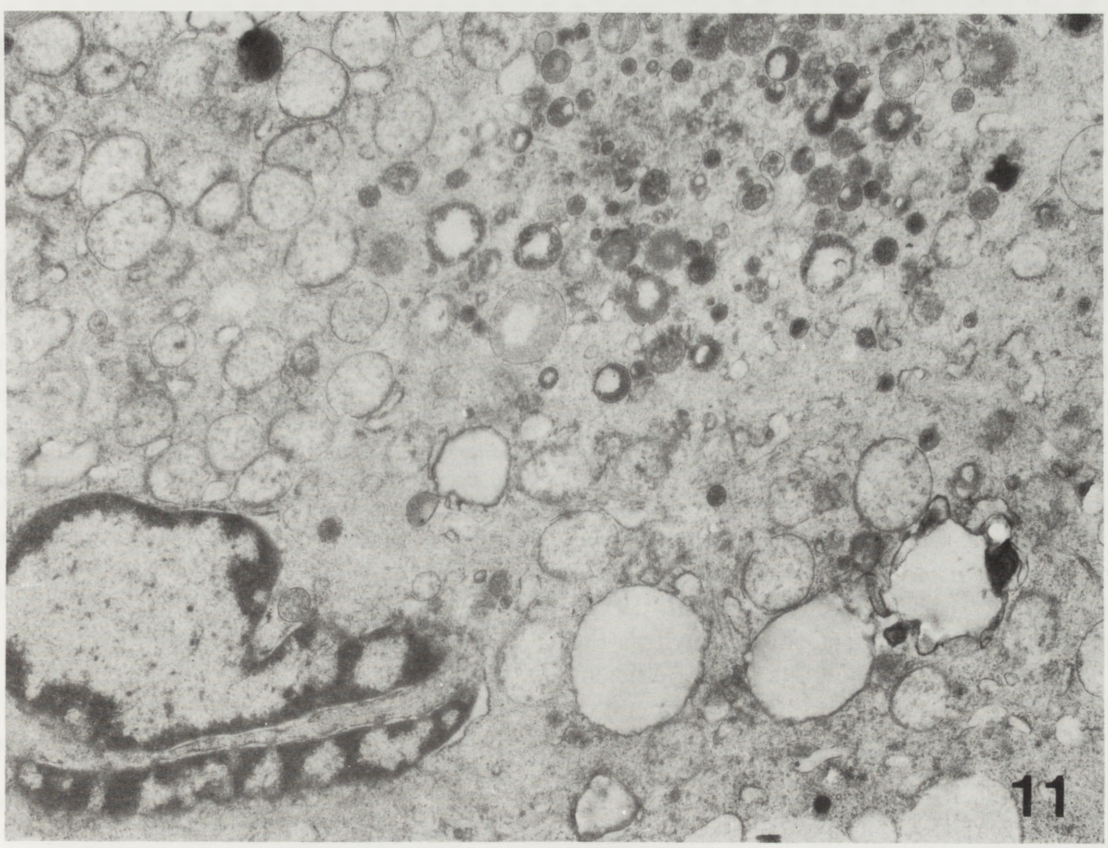

Fig. 11. Glioblastoma 18 DIV, 10 days of exposure to 10 μM 2-BdA. Tumor cell with abundant cytoplasm containing numerous damaged mitochondria, vacuoles and dark bodies. Clumping of nuclear chromatin beneath the nuclear membrane. $\times$ 17 000

Recently summarized studies of the same author (Hoshino 1992) led to the conclusion that growth fraction of gliomas (i.e., the fraction of cells which are cycling) ranges 0.08 to 0.46.

Although in gliomas *in vivo* cell loss is considerable, it apparently does affect neither "truly", nor "potentially" clonogenic cells. It has been suggested some time ago (Hoshino et al. 1972) that tumor growth in GBM is depressed by cell crowding, and some permanently resting cells (i.e., cells "frozen" in the G_0 phase of the cell cycle) may be recruited to the proliferating pool after a partial surgical removal of the tumor. This may explain the failure of "conventional" chemotherapy, which preferentially targets cycling cells, but it misses resting cells among which some may have potential to become clonogenic. Therefore, albeit for a different reason, a chemotherapy directed against non-dividing cells may prove as useful in gliomas, as it is in indolent leukemias/lymphomas.

In our organotypic cultures exposed to 2-CdA or 2-BdA the electron-microscopic picture of apoptosis was sparsely encountered. This observation is compatibile with our previous suggestion (Grieb et al. 1995) that in dissociated cultures cells are most prone to enter 2-CdA-induced apoptosis during the S phase of the cell cycle. The incidence of mitoses in organotypic glioma cultures is low, resembling that seen in gliomas *in vivo* (Hess et al. 1983).

A novel, and potentially important, finding of the present study is that in micromolar concentrations and after prolonged exposure 2-CdA, as well as 2-BdA, induced severe mitochondrial damage, mostly confined to low-differentiated cells (some of which may be noncycling, but "potentially" clonogenic). Although the functional significance of this damage is not known, one may expect that mitochondrial swelling and the disappearance of cristae may signal disturbed cellular energy metabolism leading to decreased cell viability.

Mitochondria contain deoxyguanosine activity which is able to phosphorylate 2-CdA locally (Eriksson et al. 1994), and they may be a direct target for toxicity of halogenodeoxyadenosines. In glioblastoma cells increased number of mitochondria, their enlargement and presence of atypical cristae were noted (Luse 1960; Tani et al. 1971). Underlying biochemical changes (not known at the moment) could perhaps result in more efficient phosphorylation of 2-CdA and 2-BdA in mitochondria of less differentiated cells, leading to more pronounced inhibition of mitochondrial DNA synthesis by the activated drugs.

Of notice is also, that at the same molar concentration and exposure time 2-CdA and 2-BdA apperaed to inflict similar degree of mitochondrial damage in glioma anaplastic cells. This is contrast with cytotoxicity assays on lymphoblastoid cell lines (Kazimierczuk et al. 1990), which showed that, on a molar basis, 2-CdA is three to ten times more potent than 2-BdA. However, cytostatic effect in lymphoblastoid cells is related to interference of halogenodeoxyadenosine triphosphates with nuclear DNA replication.

From the results of the present study it is evident that toxic effects of 2-CdA against nondividing glioma cells can be expected at concentrations and exposure times far above the limit set by the maximal tolerated dose of the drug upon systemic administration. In the later case nanomolar, not micromolar concentrations are encountered in plasma (Liliemark, Juliusson 1994); further dose escalation leads to bone marrow toxicity and peripheral neuropathy (Beutler et al. 1991). This limitation may be circumvented by local delivery of the drug, eg. *via* an implanted biodegradable sustained release polymer. Such local drug-delivery systmes, providing sustained high drug levels in brain tissue at the tumor site, are under development for other cytotoxic drugs (Yang et al. 1989; Judy et al. 1995), including clinical trials (Brem et al. 1991). On the basis of our data the development of a local delivery system for 2-CdA is indicated.

Zmiany w mitochondriach po ekspozycji organotypowych hodowli ludzkich złośliwych guzów glejopochodnych na 2-chloro i 2-bromo-2'-dezoksyadenozynę

Streszczenie

Badaliśmy efekty ekspozycji organotypowych hodowli ludzkich złośliwych guzów glejopochodnych na 2-chloro-2'-dezoksyadenozynę (cladribine, 2-CdA) i pokrewną jej 2-bromo-2'-dezoksyadenozynę (2-BdA). Hodowle wyprowadzone zostały z biopsji chirurgicznych 10 glejaków. W sześciu przypadkach były to glioblastoma, w trzech anaplastyczne gwiaździaki, a w jednym gwiaździak włóknisty o niskiej złośliwości. Po 6–10 dniach wzrostu *in vitro* hodowle były inkubowane z 2-CdA lub 2-BdA w stężeniach od 0,3 do 10 μM przez 1 do 10 dni. Zaobserwowaliśmy zależne od dawki i czasu ekspozycji obrzmienie mitochondriów i zanikanie grzebieni mitochondrialnych. Objawy cytotoksyczności wobec mitochondriów występowały jedynie w wysoko anaplastycznych (nisko-zróżnicowanych) komórkach. Nie stwierdziliśmy różnic pomiędzy efektem 2-CdA i 2-BdA.

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