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Research Article

Cytotoxic activity of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide is underlain by DNA interchain cross-linking

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Abstract. Currently, chemical bifunctional cross-linkers are regarded as promising therapeutic agents capable of affecting cell metabolism. Depending on the nature of the active groups and on the length of their mediating spacer, these cross-linkers have been shown to influence mitochondrial functions, the cell cycle and cell death. The current study was aimed to assay cellular effects of a cross-linker with 'zero'-length spacer, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). When added to cultures of transformed cells, EDC induced a G2/M blockade followed by cell death. Analysis of the molecular targets revealed that alteration of the cell cycle was caused by EDC-induced interchain cross-linking within double-stranded DNA. Administration of EDC to animals with experimental tumors increased their life span. The analysis of tumor cells from EDC-treated mice showed up-regulation of p21/WAF1, disturbance of tumor cell cytokinesis and, hence, cell death. Thus, both *in vitro* and *in vivo*, EDC exhibits cytotoxic activity, which may be of potential therapeutic use.

Key words. Cross-linker; apoptosis; necrosis; cell cycle; antitumor activity.

Treatment of living cells with certain chemical cross-linkers is known to cause drastic changes in cell metabolism. Specifically, cross-linkers affect mitochondrial functions [1], the cell cycle [2, 3] and the cell death process [1–4]. Despite wide recognition of chemical cross-linkers in experimental biochemistry, their cytotoxic properties remain unclear.

Our previous studies have shown that cross-linkers with a 10 to 12-Å spacer, dimethyl suberimidate dihydrochloride (DMS) and 1,5-bis(succinimido-oxycarbonyloxy)-pentane (BSOCOP), induce apoptosis by engaging various signal pathways, and affect the efficiency of tumor necrosis factor (TNF) dependent and Fas-mediated cell death [5]. The current paper describes cellular effects of a cross-linker with 'zero'-length spacer, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). This membrane-permeable bifunctional cross-linker irreversibly binds vicinal free amino or hydroxyl groups to acidic groups (including carboxyl or hydrophosphate ones). Upon addition to various cultures of transformed cells, EDC induced a G2/M blockade followed by cell death. Analysis of the potential intracellular targets of EDC revealed its ability to cause DNA interchain cross-linking. The fatal character of this damage provokes a delay in the cell cycle, and cell death. Administration of EDC to animals with experimental tumors resulted in the disturbance of tumor cytokinesis, followed by cell death and, consequently, in the increased lifetime of experimental animals.

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Materials and methods

Materials. The reagents used were from Fluka, Gibco BRL, Sigma and Laverna. EDC was supplied by Serva.

Animals. DBA/2 male mice with a body mass of 20–22 g and immunologically normal wild-type male mice with a body mass of 22–25 g were received from the Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences.

Cell cultures. The following cell cultures were used: U-937 human histiocytic lymphoma cells, K562 human chronic myelogenous leukemia cells, human Burkitt's lymphoma Raji cells, MCF7 human breast adenocarcinoma cells, human cervical adenocarcinoma HeLa cells, CV-1 kidney cells from a small African green monkey, BALB/c NS0/1 murine myeloma cells, and L929 cells of murine fibroblasts. The MCF7 cell line was kindly provided by Dr. S. A. Soukharev (NIH, USA). Other cell lines were from the Specialized Collection of Continuous Cell Lines of Vertebrates (Institute of Cytology, Russian Academy of Sciences). The cells were grown in DMEM (L929, HeLa, MCF7, CV-1) or RPMI-1640 medium (U-937, K562, Raji, NS0/1) supplemented with 10% fetal serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% CO_2-humidified atmosphere at 37 °C.

Cell treatment with EDC. EDC was dissolved in serum-free culture medium (pH 8–8.5) immediately before its addition to cells. Cells were washed twice with serum-free culture medium, supplemented with the cross-linker, and incubated for 1 h; then, fetal serum was added to a final concentration of 10%, and incubation was continued for the indicated time.

Cytotoxicity assay in vitro. For a cytotoxic analysis, the cells were seeded in 96-well microtiter plates at a density of $2–3\times10^5$ cells/ml. Cell viability was determined by MTT staining [6] and trypan blue dye exclusion assay. After treatment with EDC, the cells were incubated for 24 h. MTT diluted with cell culture medium to a final concentration of 1 mg/ml was added to each well. After the staining had developed, the medium was removed, formazan crystals were dissolved in dimethyl sulfoxide, and staining intensity was determined spectrophotometrically at 540 nm. Viability of EDC-treated cells assessed by the MTT test was expressed as a percentage of the untreated controls; in the case of trypan blue staining, as a percentage ratio between trypan blue-stained cells and their total amount.

DNA analysis. DNA fragmentation in dying cells was analyzed by 1.5% agarose gel electrophoresis as described earlier [7].

DNA treatment with EDC. Immediately before its addition to DNA, EDC was dissolved in 10 mM Tris-HCl, pH 7.5. DNA samples of plasmid (pcDNA3) or genomic (from U-937 cells) origin were incubated with 100 μM EDC for 1 h, denaturated, and separated in a 1.4% agarose gel under denaturating conditions [8].

Cytofluorometric analysis. For a cytofluorometric analysis, cells were fixed by 50% ethanol and stored at –20 °C. Prior to the analysis, cells were precipitated by centrifugation, the supernatant was removed, and the pellet was resuspended in solution containing 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.4, with 1 μg/ml of the DNA-specific fluorescent probe Hoechst-33258. The staining was carried out at 37 °C for 20–40 min.

Immunoblotting. Protein expression was studied using cell extracts yielded by cell lysis in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1 mM sodium vanadate and 10 μg/ml solutions of leupeptin, pepstatin, and aprotinin. The protein concentration was assayed according to Bradford [9]. After electrophoresis in 12% SDS-PAGE [10], the samples were transferred onto a nitrocellulose membrane. For immunoblotting, anti-p21 monoclonal antibodies were used as primary antibodies, and anti-murine antibodies conjugated with peroxidase were used as secondary antibodies.

***In vivo* antitumor activity assay.** Groups of ten animals were formed to study EDC antitumor activity. To induce tumors, at day 0, DBA/2 mice received P388 lympholeukemic cells (1×10^6), and wild-type mice were injected with Ehrlich ascite carcinoma (EAC) cells (1×10^7). An EDC dose of 15 mg/kg weight was injected intra-abdominally into the mice of experimental groups at days 1, 4, and 7. In control groups, mice were injected with physiological salt solution according to the treatment scheme. The results were assessed by the average life span (in days) of animals from experimental groups compared with the controls, as well as by inhibition of the rate of tumor growth; the latter was followed by measuring tumor cells in mice with EAC. The EDC antitumor activity was defined as a percentage ratio between the average life span of animals from the experimental groups and that of the controls [11]. The statistical processing of the results was performed using the Student t criterion.

Results and discussion

EDC effects *in vitro*. In the first set of experiments, the effect of EDC on the viability of the various transformed cells, specifically on human U-937, Raji, K562, MCF7, HeLa, murine L929, NS0/1 and monkey CV-1 cells was

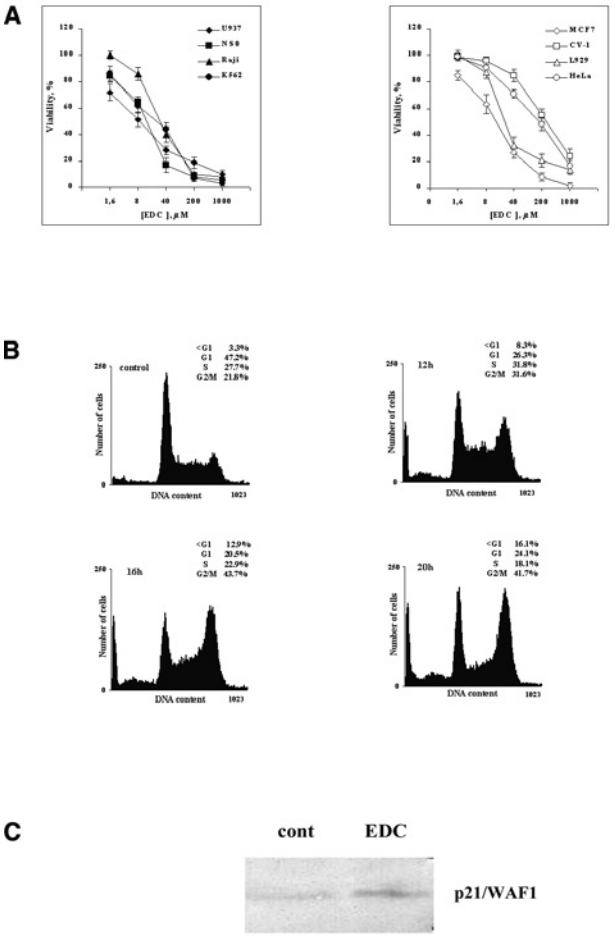

Figure 1. Effect of EDC on cultured cells. (*A*) EDC-induced inhibition of growth of various cells. U937, NS0, Raji and K562 cells as well as MCF-7, CV-1, L929 and HeLa cells were incubated with the indicated EDC concentrations for 24 h, and their viability was estimated by MTT assay. Values shown are the mean ± SE of three independent experiments. (*B*) EDC induces G2/M arrest followed by apoptosis. Cell cycle distribution of U-937 cells incubated in the presence of 8 μM EDC for the indicated times was analyzed by FACS. (*C*) Western blotting of the total proteins of U-937 cells (control and treated with 8 μM EDC for 16 h), using anti-p21 mABs.

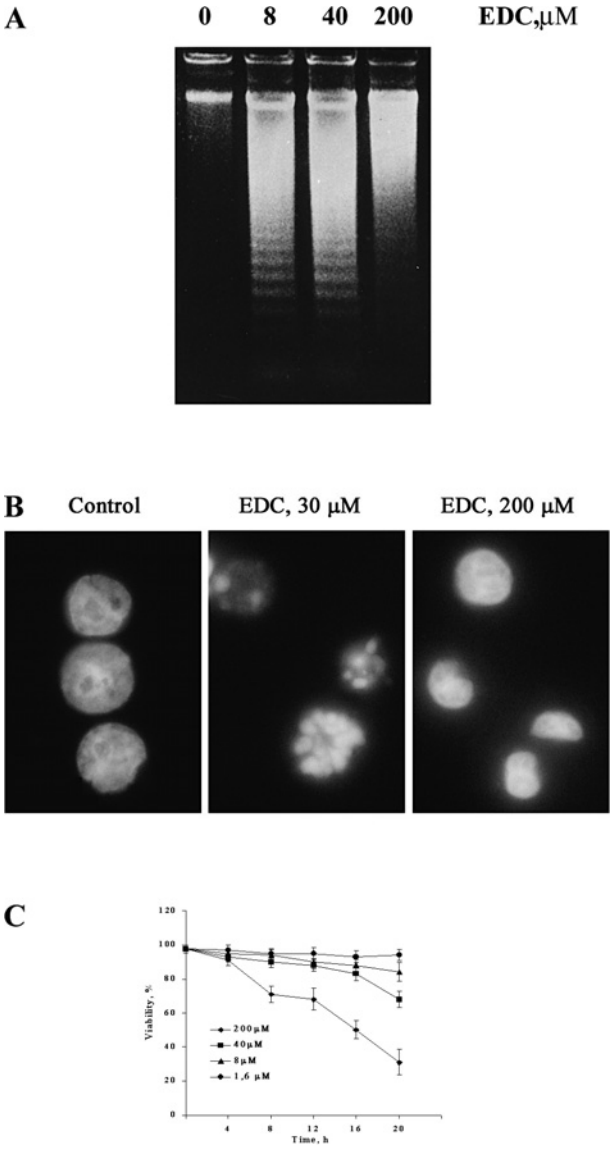

Figure 2. Dose-dependence of the EDC-induced cell death type: apoptosis or necrosis. (*A*) U937 DNA fragmentation induced by various doses of EDC. Cells were treated with 8 and 40 μM EDC for 20 h and analyzed for the appearance of oligonucleosomal DNA fragments as described in Materials and methods. (*B*) Fluorescent microscopy analysis of U937 cells treated with EDC of various concentrations for 20 h and stained with Hoechst 33258. (*C*) U937 cell death induced by various doses of EDC. Cell viability was estimated by trypan blue exclusion assay.

studied. As seen in figure 1A, incubation with EDC within the concentration range 2–200 μM caused inhibition of growth in all studied cultures. This inhibition could result from both cytostatic and cytotoxic activity of EDC. To reveal the EDC effect on the cells, we studied the distribution of EDC-treated U-937 cells over the cell cycle. As soon as 12 h after exposure to EDC, the number of cells in the G1 phase decreased markedly, whereas S- and G2/M cell fractions increased significantly. This points to a cell hold-up at the S phase and to a G2/M blockage (fig. 1B). Cell retardation in the G2/M fraction was accompanied by increased expression of p21/WAF1, a universal inhibitor of cyclin-dependent kinases [12–14] (fig. 1C). In addition, as soon as 12 h after incubation with EDC, the first dying cells appeared and their fraction increased with time. Thus, the cytotoxic effect of EDC is coupled to its cytostatic activity.

Morphological analysis of the nuclei of dying U-937 cells and electrophoretic separation of DNA from these cells were used to determine the type of EDC-induced cell death (apoptosis or necrosis). Electrophoretic analysis of DNA isolated from U-937 cells treated with 8 and 40 μM EDC for 20 h showed an apoptotic 'ladder' of DNA fragments corresponding to nucleosomes and their oligomers

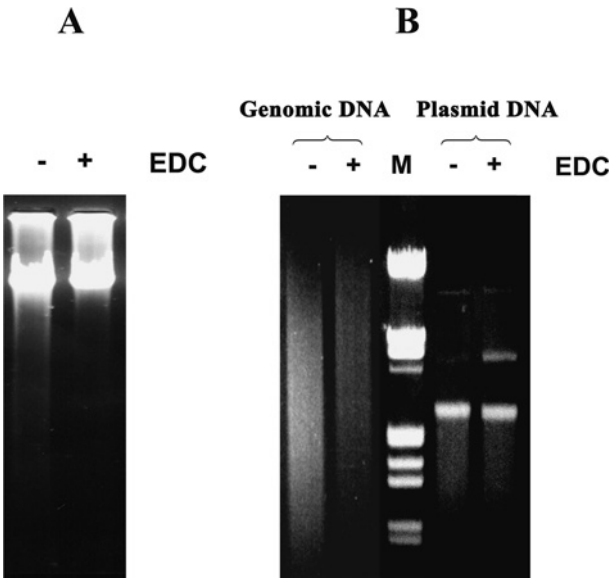

Figure 3. EDC causes interchain cross-linking within DNA. (*A*) DNA isolated from control and EDC-treated cells. Cells were treated with 100 μM EDC for 3 h. Electrophoretic analysis was performed under denaturating conditions. (*B*) Samples of genomic (10 μg) and plasmid (3 μg) DNA were incubated with 100 μM EDC for 1 h, denaturated, and separated in an agarose gel under denaturating conditions, as described in Materials and methods. M, molecular weight markers.

(fig. 2A). DNA from dying cells exposed to a higher EDC concentration (200 μM) gave a 'smear' (fig. 2A) typical for cells dying by necrosis [15, 16].

Morphological analysis of dying cells also revealed that moderate EDC doses induced apoptosis, while high EDC concentrations resulted in necrosis (fig. 2B). DNA-specific Hoechst 33258 staining of U-937 cells treated with 20 μM EDC showed that, unlike the control, the nuclei of dying cells had typical apoptotic features, that is, condensed chromatin and nucleus fragmentation. In contrast, higher concentrations of EDC (200 μM) induced typical necrotic swelling of U-937 cells after 12 h: their nuclei remained non-fractionated, slightly shrunk and showed neither chromatin condensation nor its marginal localization. It should be noted that, in contrast to cells exposed to low EDC concentrations, the plasma membrane of 200 μM EDC-treated cells was trypan blue permeable (fig. 2C). The above results strongly suggest that low or moderate concentrations of EDC induce U-937 cell death by apoptosis, whereas high concentrations cause necrotic cell death.

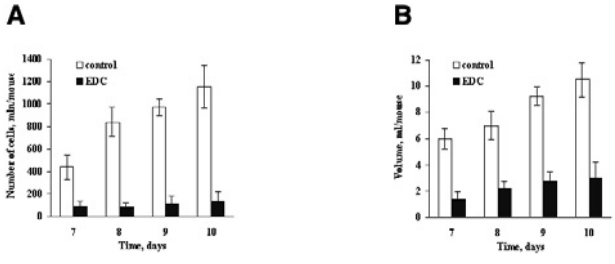

Figure 4. EDC treatment inhibits tumor cell growth in the peritoneum. Wild-type mice were inoculated with 1×10^7 Ehrlich ascite carcinoma cells per mouse and injected with PBS (control) or EDC (15 mg/kg weight) at day 1, 4 and 7. Peritoneal samples taken at the indicated days were used to estimate the number of cells (*A*) and the volume of ascitic fluid (*B*).

Table 1. Antitumor effects of EDC on murine ascitic tumors.

Tumors	Treatment	Median survival time (days)	Antitumor activity (%)
P 388	PBS	9.7±0.7	–
P 388	EDC	12.0±2.3	124.1*
EAC	PBS	11.1±1.2	–
EAC	EDC	14.8±1.9	133.3**

Groups of DBA/2 mice and NMRI mice (n=10) were inoculated with 1×10^6 P388 lympholeukemic and 1×10^7 EAC cells per mouse, respectively. 1, 4, and 7 days later they were injected with PBS (control) or EDC (15 mg/kg of weight), and their death was followed daily.*$p<0.05$, **$p<0.01$ (Student's t test).

Another set of experiments was aimed to reveal molecular targets of EDC. Potentially, these could be proteins, as well as nucleic acids. To detect possible protein targets of EDC, we analyzed alterations in protein spectra of U-937 and other transformed cell lines exposed to 100 μM EDC for 3 h. The distribution of total proteins of cytoplasmic and nuclear fractions in EDC-treated cells proved to be identical to that of the control. The comparison between control and EDC-treated cell lysate fractions separated by ion chromatography (MonoQ and MonoS) or gel filtration (Sepharose 12) with subsequent SDS-PAGE electrophoresis also revealed no difference between them (data not shown).

Electrophoretic analysis of native DNA from control and EDC-treated (100 μM EDC for 3 h) cells also revealed no differences. However, separation performed under denaturating conditions showed that migration of control DNA was faster than that of EDC-treated cells (fig. 3A). The retardation of the latter could be caused either by DNA-protein cross-linking or by cross-linking within DNA [17–20]. Further experiments on purified plasmid and genomic DNA revealed that EDC induced interchain cross-linking within the double-stranded DNA (fig. 3B). Thus, the fatal derangement of genomic DNA could be one of the reasons for EDC-induced cell cycle perturbations followed by apoptosis.

Effects of EDC in vivo. Further experiments were performed to study the cytotoxic effects of EDC *in vivo* using mice with inoculated Ehrlich ascite carcinoma (EAC) and P388 lympholeukosis. An EDC dose of 15 mg/kg weight was administered intra-abdominally to mice from the experimental groups at day 1, 4 and 7. In the control group, mice were injected with physiological

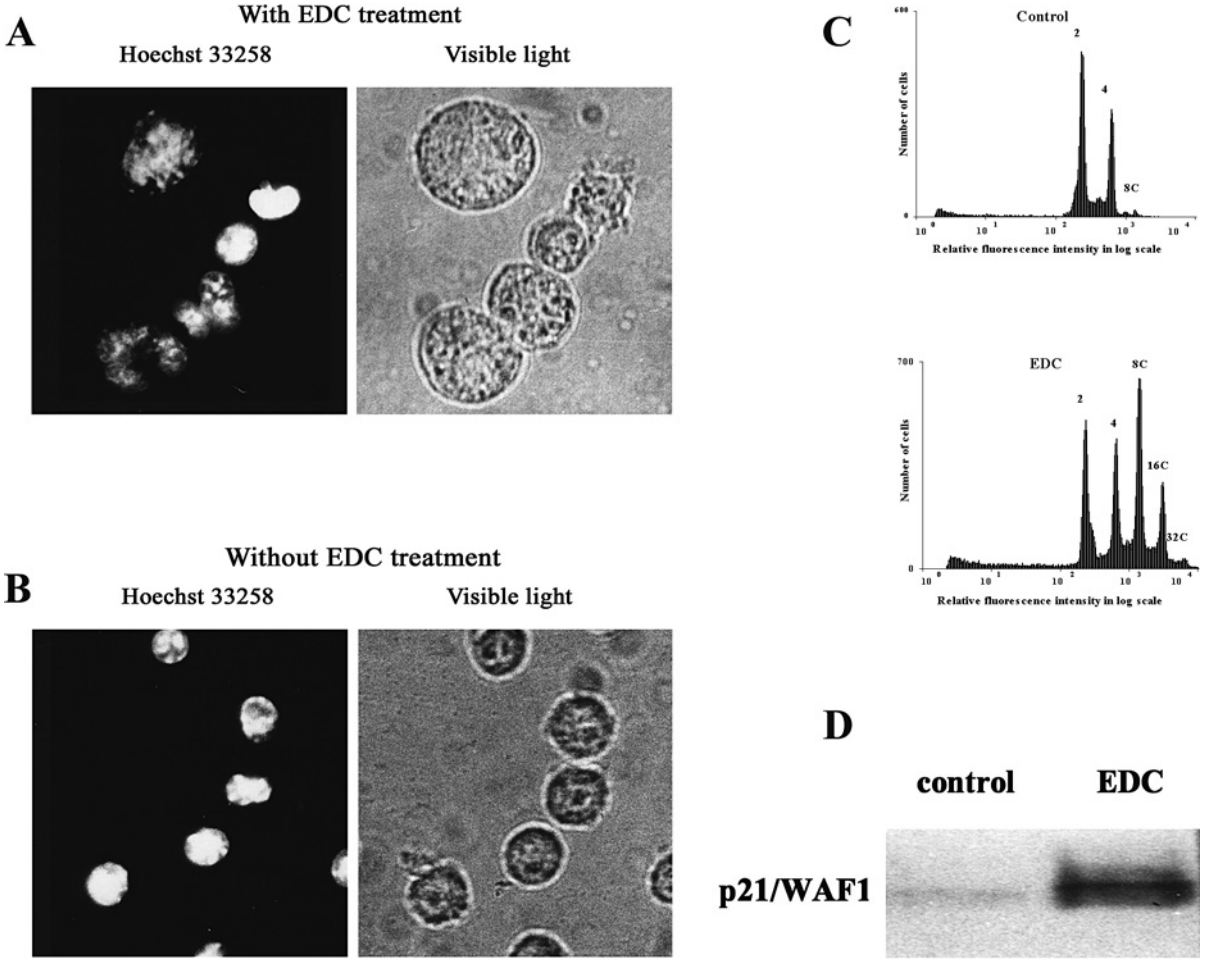

Figure 5. EDC blocks EAC cell cytokinesis resulting in the appearance of multinuclear cells. (*A*) EAC cells isolated from EDC-treated mice at day 9 were stained with Hoechst 33258. (*B*) EAC cells isolated from non-treated mice at day 9 were stained with Hoechst 33258. (*C*) FACS analysis of EAC cells isolated from control and EDC-treated mice at day 9 (for details of the experiment, see fig. 4 legend). DNA content was estimated as relative fluorescence intensity on a log scale. (*D*) EDC up-regulates expression of p21/WAF1 in EAC cells. Western blotting of the total proteins (50 µg) of EAC cells isolated from PBS-treated and EDC-treated animals at day 9 was performed using anti-p21 monoclonal antibodies.

salt solution on the same days. Tumor proliferation in EDC-treated animals differed strongly from that in controls. EAC and P388 tumor nodules appeared 4 and 5 days after inoculation, respectively. Further growth of the EAC and P388 tumors resulted in animal death after 10 and 12 days. EDC caused a strong decrease in the amount of tumor cells and ascitic fluid in the animals (fig. 4). Seven to ten days after the inoculation, the amount of tumor cells in EDC-injected mice was about an order of magnitude less than in the control, and the volume of ascitic fluid decreased two- to threefold. As shown in table 1, the life span was extended in the both experimental groups, compared with the control. The EDC antitumor activity was 124% for the mice with P388 and 133% for the mice with Ehrlich carcinoma. An effect of EDC on normal mice survival was not observed.

The fluorometric analysis of cells isolated from the peritoneal cavity of EDC-treated animals showed that tumor cells were larger than control cells (fig. 5A, B). Moreover, the overwhelming majority of the isolated cells contained two or more nuclei. Flow cytofluorometry showed that 25% and 10% of EAC cells isolated from EDC-treated animals contained 8C and 16C DNA, respectively (fig. 5C). The above results indicate that EDC causes disturbance of cytokinesis in EAC cells.

One of the possible reasons for polynuclear cell accumulation could be an enhanced expression of p21/WAF1/CIP1 [12–14]. To define the role of p21/WAF1/CIP1 in EDC effects in the cell, Western blot analysis using specific anti-p21 antibodies was performed. EDC treatment caused an enhanced expression of p21/WAF1/CIP in EAC cells (fig. 5D), as in the case of *in vitro* treatment of U-937 cells (fig. 1B). Thus, the enhanced expression of p21/WAF1/CIP that causes disturbance of cytokinesis followed by cell death most likely results from EDC-induced DNA derangement. The absence of dead cells in

the animal peritoneal cavity can probably be explained by their phagocytosis by neutrophils and macrophages present at the inflammation locus.

Taken together, the results reported here show that both *in vitro* and *in vivo* EDC displays a cytotoxic activity resulting from interchain cross-linking within DNA. This disturbs the regulation of the cell cycle at the stage of cytokinesis, which leads to selective death of rapidly dividing cells. The compound is believed to be effective not only against ascitic carcinoma but also against other types of tumor cell. Furthermore, we suggest that EDC can enhance the antitumor activity of known antitumor agents (G2/M-specific ones among them), if jointly administered. Further experiments in these directions are in progress.

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