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L-Asparaginase kills lymphoma cells by apoptosis*

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Abstract. Microscopic examination of histological sections of lymph nodes from a canine case of malignant lymphoma at 4 h after treatment with L-asparaginase revealed massive destruction of neoplastic cells by what was consistent with apoptosis morphologically. Apoptosis as the mode of cell death after asparaginase treatment was confirmed in a mouse lymphoma cell line (LY-TH) by the characteristic fragmentation of DNA into oligonucleosome-sized pieces and by the morphological changes consistent with apoptosis following treatment in vitro. Applied to these cells, asparaginase was found to be most cytotoxic over the range of 1–10 IU/ml. Even after 4 h of asparaginase treatment at 100 IU/ml, protein synthesis was reduced by only one-half, yet DNA fragmentation reached 40%. Other agents that affect protein synthesis (cycloheximide and actinomycin D) caused apoptosis as well; however, agents (radiation, prednisolone, and VP-16) whose mechanisms are different from inhibition of protein synthesis also caused apoptosis. As such, it seems unlikely that protein depletion per se and/or the elimination of specific short-lived proteins is the triggering event that leads to cell death. It is more likely that the suspension of cellular proliferation commits cells to apoptosis after asparaginase treatment.

Introduction

Even though asparaginase has been used successfully for many years as a chemotherapeutic agent in the treatment of acute lymphocytic leukemia and lymphomas [2, 5, 7], its mechanism of action is not completely understood. The cytotoxicity seen in tumor cells as a result of asparaginase exposure has been attributed to its ability to inhibit protein synthesis by eliminating the extracellular pools of asparagine through the conversion of asparagine to aspartate [11] or an inhibition of basic energy mechanisms by the accumulation of aspartate [3]. Accordingly, cells that cannot synthesize asparagine de novo are sensitive to this therapy, whereas cells that are capable of up-regulating asparagine synthase in response to depletion of exogenous asparagine are refractory [7, 11]. Lymphoid cells in general are known to be susceptible to other less specific protein-synthesis inhibitors such as cycloheximide and actinomycin D. Exposure to such agents at doses that need not completely inhibit protein synthesis can nonetheless result in cell death, and in these cases the mode of death usually involves apoptosis [4, 9, 18]. Apoptosis is distinctly different from necrosis or reproductive cell death; rather, it is characterized by chromatin condensation, pyknotic nuclei, cytoplasmic condensation, and the cleavage of chromatin DNA into oligonucleosome-sized fragments by a putative Ca^{2+}/Mg^{2+}-dependent endonuclease (see [15, 20] for a review). As shown in this report, examination of the cellular morphology of a lymphoma in a dog both before and 4 h after asparaginase therapy as well as LY-TH cells treated in vitro determined the mode of cell death caused by asparaginase exposure to be apoptosis.

Materials and methods

Canine lymphoma. The left popliteal lymph node of a male 5-year-old beagle was excised before treatment, whereas the right popliteal lymph node was excised at 4 h after the intraperitoneal administration of 20,000 IU/m^2 L-asparaginase (Elspar; Merck, Sharp & Dohme, West Point, Pa.). The lymph nodes were fixed by immersion in phosphate-buffered 10% formalin solution. Following processing and embedding in paraffin, sections were cut and stained with hematoxylin and eosin.

Cell culture and irradiation. LY-TH cells derived from a mouse B-cell lymphoma were maintained in culture in RPMI medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 1% L-glutamine at 37° C in an atmosphere of 5% CO_2 in air. Under these condi-

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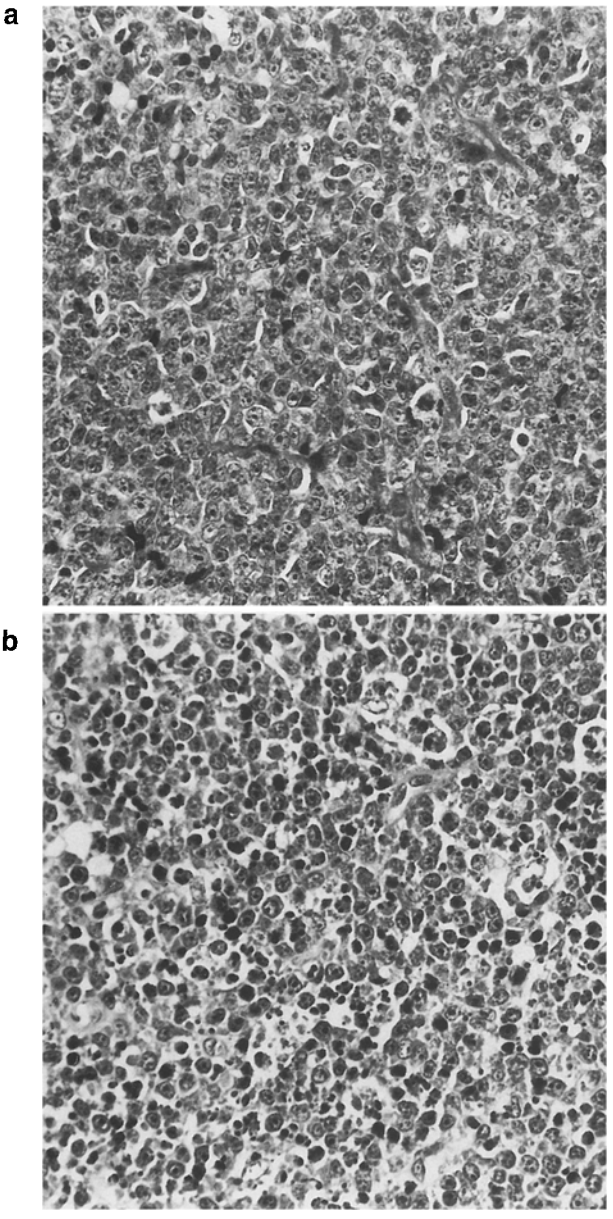


Fig. 1 a, b. Histological sections of a canine malignant lymphoma involving popliteal lymph nodes. **a** Pretreatment. **b** At 4 h after L-asparaginase treatment there is extensive apoptosis of the neoplastic lymphoid cells. H&E; original magnification, ×400

tions the cells doubled approximately every 11 h. Before treatment, cells were radiolabeled over a 12-h period with medium containing 0.033 μCi [^{14}C]thymidine/ml followed by a 1 h chase in medium without radiolabel. In some experiments, cells in 37° C medium were irradiated at room temperature using a ^{137}Cy γ-ray source at a dose rate of approximately 4.5 Gy/min as a positive control for apoptosis induction in these cells.

DNA-fragmentation analysis. The method for quantifying DNA fragmentation was applied as described elsewhere [18], with minor modification. Briefly, after a wash with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; Irvine Scientific, Santa Ana, Calif.), 10^7 cells were lysed in a solution of 10 m*M*, 1 m*M* ethylenediaminetetraacetic acid (EDTA), and 0.2% Triton-X 100 for 20 min while being maintained at 4° C. Chromatin-size DNA remained insoluble, whereas oligonucleosome-sized DNA fragments were soluble in the low-salt-concentration lysis buffer. The

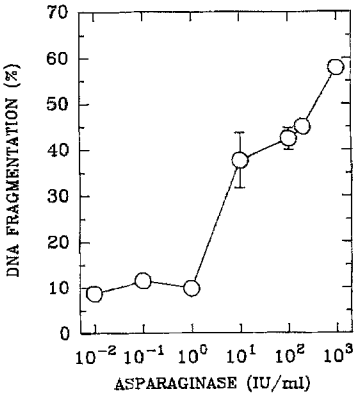


Fig. 2. DNA fragmentation in LY-TH cells as a result of 4 h of continuous exposure to increasing concentrations of asparaginase. Error bars represent standard errors from 3 separate experiments

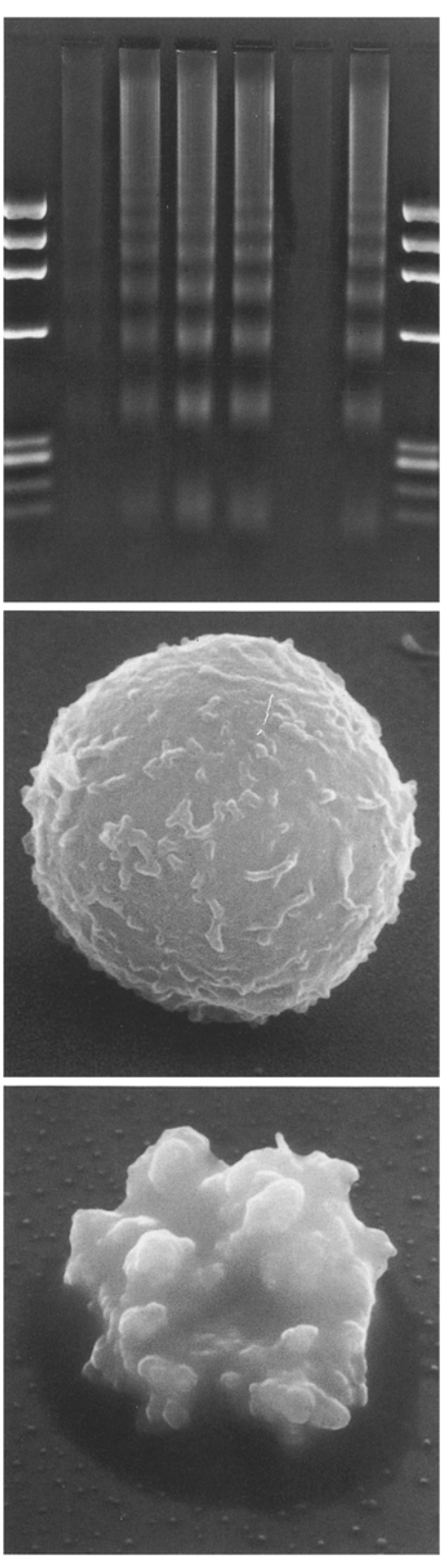
chromatin-sized DNA fragments (pellet) were separated from the soluble fragments (supernatant) by centrifugation at 13,000 *g* for 10 min. The DNA in the separate pellet and supernatant samples was then precipitated in 12.5% trichloroacetic acid (TCA) overnight at 4° C. The precipitated DNA was transferred to a liquid scintillation vial with 0.3 ml H_2O to which 1 ml Solulene (Packard, Downers Grove, Ill.) was added. After incubation at 60° C for 1 h, 15 ml liquid scintillation fluor was added and the radioactivity was counted in a liquid scintillation counter. DNA fragmentation was expressed as the amount of radioactivity detected in the supernatant fraction as compared with the total amount found in both the supernatant and pellet fractions.

Gel electrophoresis. As in the DNA-fragmentation assay, the cells were lysed and the fragmented DNA of the supernatant sample was separated from the pelleted chromatin-size DNA. However, the DNA in the supernatant sample was instead precipitated in a solution of 0.5 *M* NaCl in 50% isopropanol overnight at –20° C. The following day the precipitate was collected by centrifugation at 13,000 *g* for 10 min and allowed to air dry. The precipitate was resuspended in TE buffer (10 m*M* TRIS, 1 m*M* EDTA, pH 7.5), incubated for 30 min with 2 mg RNAse/ml at 50° C, and then loaded into a 1.75% (w/v) agarose gel. The DNA was electrophoresed (3.7 V/cm) over 3–4 h at room temperature in 1X TBE buffer (89 m*M* TRIS, 89 m*M* boric acid, 2 m*M* EDTA, pH 8.3). The gel was stained with ethidium bromide, and the DNA fragments were visualized and photographed after illumination by UV light.

Protein synthesis. New protein synthesis was determined by measuring the incorporation of [^{3}H]-leucine into protein following the addition of 50 μCi to 5 ml medium containing 5×10^6 cells. The cells were radiolabeled for 30 min followed by a 30-min chase in nonradioactive medium. Following a PBS wash, the cells were collected and the radioactivity was counted in the same manner described for the DNA-fragmentation assay above.

Results and discussion

Familiarity with the morphology of apoptosis gained from the study of murine tumors and a recent determination that the peak of the apoptosis response occurs within 3–6 h of irradiation in normal thymocytes [18] and murine lymphomas (unpublished results) reminded us of a canine case of malignant lymphoma treated with asparaginase that first presented in 1982. L-Asparaginase is commonly used as a single agent or in combination with other chemotherapy agents for the induction and maintenance of remission of



malignant lymphoma in dogs [13, 14]. Cases of canine lymphoid neoplasia commonly present with a peripheral lymphadenopathy that often responds rapidly to the administration of L-asparaginase as the affected lymph nodes shrink dramatically. As apoptosis is a peracute response to radiation and drug exposures (cycloheximide, actinomycin D, glucocorticoids) in some murine tumors [17] and excised rat thymocytes [18], we were fortunate in that this dog had had excisional biopsies of peripheral lymph nodes taken before and at 4 h following asparaginase administration, making it possible to determine the morphological effects of treatment. The pretreatment specimen had the normal architecture diffusely replaced by sheets of medium-sized lymphoid cells. The cells had scanty cytoplasm and ovoid nuclei that contained single or multiple amphophilic nucleoli (Fig. 1 a). The histology of the second specimen differed markedly from that of the first. In this lymph node, the monotony of the sheets of neoplastic lymphoid cells was radically effaced (Fig. 1 b). Relative to intact nuclei, there were greater numbers of smaller, darkly stained structures that are characteristic of apoptotic bodies [16, 21]. The apoptotic bodies existed singly and in clusters. They typically had a narrow eosinophilic rim surrounding a homogeneous basophilic nuclear fragment that had a variety of configurations, including pyknotic, karyorrhexic, and crescent forms. Both intracellular and extracellular apoptotic bodies were observed.

Given the extensive appearance of apoptotic cells in the tissue sections of the asparaginase-treated canine lymphoma, it was decided to study asparaginase-induced apoptosis in a well-characterized mouse lymphoma cell line, LY-TH. These cells, originally isolated from a mouse B-cell lymphoma, undergo apoptosis in response to a variety of stimuli, including radiation, whereby a 5-Gy exposure results in 70% DNA fragmentation at 4 h after exposure.

Fig. 3. a Photograph of an ethidium bromide-stained agarose gel, depicting the DNA-fragmentation pattern of cells that have undergone apoptosis after a 4 h asparaginase exposure. *Lanes 1 and 8,* DNA size markers consisting of ΦX174 Hae III restriction fragments of sizes 1353, 1078, 872, 603, 310, 271/281, 234, and 194 bp, respectively, from the top of the lane; *lane 2,* 1 IU/ml; *lane 3,* 10 IU/ml; *lane 4,* 100 IU/ml; *lane 5,* 200 IU/ml; *lane 6,* control; *lane 7,* 5 Gy γ-irradiation. The γ-irradiated sample serves as an apoptosis-positive control and was observed at 4 h after a 5-Gy radiation exposure. **b** Scanning electron micrograph of an untreated LY-TH cell at a magnification of × 13,000. For scale, 1 cm represents 0.72 μm. **c** Scanning electron micrograph of a LY-TH cell after a 4-h exposure to 100 IU asparaginase/ml. The magnification is × 10,000 and 1 cm represents 0.94 μm

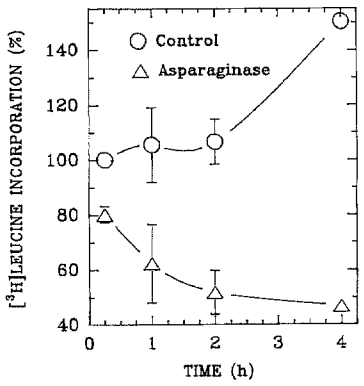


Fig. 4. [^{3}H]-Leucine incorporation in cells exposed or not exposed to 100 IU asparaginase/ml over a 4 h period. The values represent comparisons of the radiolabel incorporation at different times with the untreated control value at the beginning of the treatment period. Error bars reflect the standard deviation from 2 experiments each with replicate samples. Each value is plotted as being at the midpoint of the 30-min radiolabel exposure

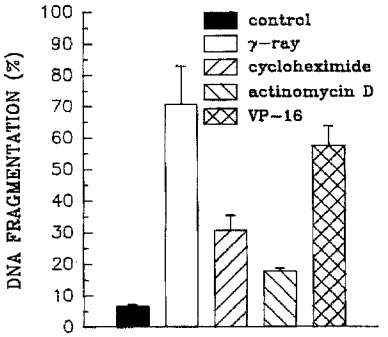


Fig. 5. DNA fragmentation in LY-TH cells measured at 4 h after continuous exposure to cycloheximide (10 μ*M*), actinomycin D (10 μg/ml), VP-16 (10 μg/ml), and 5 Gy γ-radiation. Error bars reflect standard errors from a minimum of 2 separate experiments

Because LY-TH cells are radiolabeled to determine the extent of DNA fragmentation, a nominal level of DNA fragmentation always exists inasmuch as even the radiolabel itself causes some apoptotic death. At the concentration of radiolabel used for these experiments the nominal level of DNA fragmentation was 8%. As depicted in Fig. 2, apoptosis above the nominal level was not evident until the exposure to asparaginase exceeded 1 IU/ml. Thereafter, apoptosis quickly increased as a function of the asparaginase concentration in the medium such that by 1,000 IU/ml DNA fragmentation had reached approximately 60%.

Qualitative evidence of the apoptosis induced by 4 h treatments at various asparaginase concentrations can be observed in a photograph taken of an ethidium bromide-stained agarose gel following electrophoresis of the isolated DNA (Fig. 3a) in which the oligonucleosome-sized DNA fragments characteristic of apoptotic cells can be visualized. The DNA fragments visible in lane 2, where cells received 1 IU asparaginase/ml, are comparable with those seen in lane 6, the control lane, where fragmentation is approximately 8%. The degree of fragmentation in lanes 3, 4, and 5, where the cells received 10, 100, and 200 IU asparaginase/ml, respectively, is comparable with that in lane 7, where the cells were analyzed 4 h after receiving 5 Gy radiation and the fragmentation is approximately 70%. LY-TH cells were also examined for the morphological changes associated with apoptosis. Scanning electron micrographs were made of untreated (Fig. 3b) and asparaginase-treated (Fig. 3c) LY-TH cells. The untreated cells were spherical with extensive microvilli, whereas the asparaginase-treated cells had lost their microvilli and showed extensive cytoplasmic blebbing. This blebbing, as a characteristic of cells undergoing apoptosis, has been reported previously [12].

The inhibition of protein synthesis by depletion of asparagine by asparaginase has been considered to be the biochemical event that leads to death in cells that are asparaginase-sensitive [3, 7, 11]. Recently, however, Asselin et al. [2] suggested that the asparaginase cytotoxicity observed in in vitro systems, particularly at concentrations above 1 IU/ml, may be due to a mechanism other than asparagine depletion. Whereas our data shows that the mode of cell death is apoptosis, the initiating event has not been identified. Lymphoid cell viability in the presence of certain cytokines in the growth medium may be controlled by one or more short-lived proteins whose continual synthesis is required [1, 4, 9]. Accordingly, when protein synthesis is reduced below some critical level by a protein synthesis inhibitor, the loss of these short-lived proteins propels lymphoid cells into apoptosis.

On the one hand, our data could be interpreted as being consistent with this mechanism. As depicted in Fig. 4, when LY-TH cells were treated with 100 IU asparaginase/ml over a 4-h period, which is sufficient to cause apoptosis in 40% of the cell population, the uptake of [^{3}H]-leucine was reduced by 40%. Furthermore, as shown in Fig. 5, cycloheximide and actinomycin D exposure over a 4 h period also caused apoptosis. Thus, a reduction in protein synthesis may be the critical step in the cytotoxicity of asparaginase exposure by eliminating some unknown short-lived protein(s) that, when present, block(s) apoptosis. Note, however, that the increase in protein synthesis seen in the untreated cell population was due to the increase in cell number over this period, whereas in the asparaginase-treated group the cell number remained static over the treatment period.

On the other hand, an alternative and more likely interpretation of the above data would suggest that the event that initiates apoptosis in these cells is inhibition of cell cycle progression rather than protein depletion per se. A 5 Gy radiation exposure (see Figs. 5 and 3a), which caused approximately 70% of the cells to undergo apoptosis, is not considered a protein-synthesis inhibitor but does block cell-cycle progression at the G_1/S border and in G_2 [8]. Also, as shown in Fig. 5, the glucocorticosteroid prednisolone, which causes apoptosis through an influx of Ca^{2+} [10], and VP-16, a topoisomerase II inhibitor [19], also caused apoptosis in LY-TH cells. Further evidence for interruptions of cell cycle progression as the initiator of apoptosis has been provided by Evan et al. [6], who showed that Rat-1 cells expressing the transcription factor

and oncogene *c-myc*, when growth-arrested by culturing in medium of low serum content, undergo apoptosis. Conversely, when Rat-1 cells that do not express *c-myc* are subjected to growth arrest in this manner, they remain viable for weeks in a G_0/G_1 state.

Where or how cell-cycle arrest fits into the cascade of events leading to apoptosis is unclear. Nonetheless, assays for apoptosis, including quantification of DNA fragmentation using a modified assay for cells from biopsy or blood samples that cannot be radiolabeled [18] and visualization of DNA fragmentation by gel electrophoresis, may represent a rapid diagnostic/prognostic tool whereby patients who are candidates for asparaginase therapy can be screened as to the effectiveness of this modality prior to therapy. The turnaround time for these assays is less than 48 h.

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