REVIEWS

α-Fetoprotein-Induced Apoptosis of Cancer Cells

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 130, No. 12, pp. 604-612, December, 2000
Original article submitted March 20, 1998

Apoptosis is an active physiological process of cell self-destruction induced by the activation of intracellular molecular mechanisms of programmed cell death [30]. This process is characterized by a marked decrease in cell volume, chromatin condensation, fragmentation of nuclear DNA, formation of membrane vesicles, and vacuolization of cell organelles [34]. Apoptosis plays an important role in normal and pathological cell processes [29]. The regulatory mechanism maintaining the equilibrium between cell growth, differentiation, and death is necessary for normal embryogenesis characterized by intensive cell production [13]. Cells of the central and peripheral nervous and immune systems also undergo apoptosis. Apoptosis also serves as the major cell-protecting factor: in some cases, self-destruction of infected cells followed by their elimination by macrophages is the only way to remove pathogens [29]. Suppression of apoptosis indirectly increases proliferative activity of precancer and cancer cells and aggravates autoimmune disorders [34]. At the same time, activation of apoptosis intensifies neurodegenerative processes accompanying Alzheimer's and Parkinson's diseases and leads to exacerbation of disorders induced by retroviruses (HIV-1) [8,27]. The understanding of mechanisms regulating apoptosis is an urgent problem of cell biology [31].

Apoptosis results from activation of cell receptors for tumor necrosis factor (TNF), Fas, T cells, and nerve cell growth factors [18]. On the other hand, apoptosis can be triggered without activation of membrane receptors via intracellular molecular mechanisms. This type of apoptosis is induced by radiation or active compounds (*e.g.*, antibiotics), which enter cells via a nonreceptor mechanism or through specific receptors not involved in the initiation of cell death [30].

Transduction of apoptotic signals to the cell nucleus is a series of molecular interactions, which lead to activation of various substances and specific endonucleases inducing DNA fragmentation [31]. Various proteins (TRADD, MORT, and FADD) noncovalently bound to the receptor cytoplasmic region and released after binding of Fas-antigen to the ligand Apo-1 are involved in Fas-antigen-mediated transduction of the apoptotic signals [18]. These proteins interact with specific enzymes caspases 1-10. Activated caspases 1-10 activate caspase-activated DNase (CAD) catalyzing degradation of nuclear DNA [33]. Any factor counteracting this process and blocking various stages of signal transduction can modulate apoptosis. Recent studies revealed molecular inhibitors of apoptosis (vFLIP proteins) encoded by some retroviruses, which prevent the death of infected cells [28]. Molecular inhibitors of apoptosis FLIP proteins produced by cells under certain conditions [11] and CAD inhibitors [7] were characterized.

α-Fetoprotein (AFP) is a specific fetal oncoprotein produced by normal mammalian cells during embryogenesis and expressed in cells involved in neoplastic transformation or transformed by various retroviruses [1,2]. This protein is absorbed only by activated developing cells (*e.g.*, tumor, embryonic, or immune cells) [3,5,19], because specific AFP receptors are expressed on the surface of differentiating, but not mature adult cells [15]. AFP modulates (stimulates or suppresses) growth of tumor, embryonic, and activated immune cells [9,10,12,14,16,21,23,26]. Recent studies showed that AFP induces programmed death of human tumor cells *in vitro* [24]. This protein amplifies or blocks apoptotic and activating signals po-

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0007-4888/00/0012-1127$25.00

tentiated by some cytokines [5,25]. Since AFP specifically interacts with biologically active compounds (*e.g.*, arachidonic acid and steroid hormones), its activity depends on occupation by these ligands [12,21, 22,25,26].

Here we studied the effects of human embryonic or cancer AFP on proliferative activity of cancer and normal cells and evaluated the mechanisms of AFP-induced apoptosis.

MATERIALS AND METHODS

AFP was isolated from umbilical serum by ion-exchange chromatography on DEAE-Sephacel, affinity chromatography on BrCN-Sepharose with polyclonal monospecific rabbit anti-AFP antibodies, and gel filtration through AcA-44 biogel [20,24]. The purity of AFP was not less than 99.8% (as determined by PAAG electrophoresis and immune electrophoresis). Ligand-free human AFP was obtained by treatment with activated charcoal at pH 3.5 [2]. Cancer AFP (cAFP) was isolated from cultured AFP-producing human hepatoma HepG2 cells by affinity chromatography [25].

Human hepatoma HepG2, lymphoma U-937, breast cancer MCF-7, Jurkat lymphoblastoma, MT4 cells, mouse fibroblastoma L-929 cells, and normal human skin fibroblasts M19 were used. The primary culture of embryonic fibroblasts was obtained from human lungs and retina by treatment with 0.2% trypsin. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS).

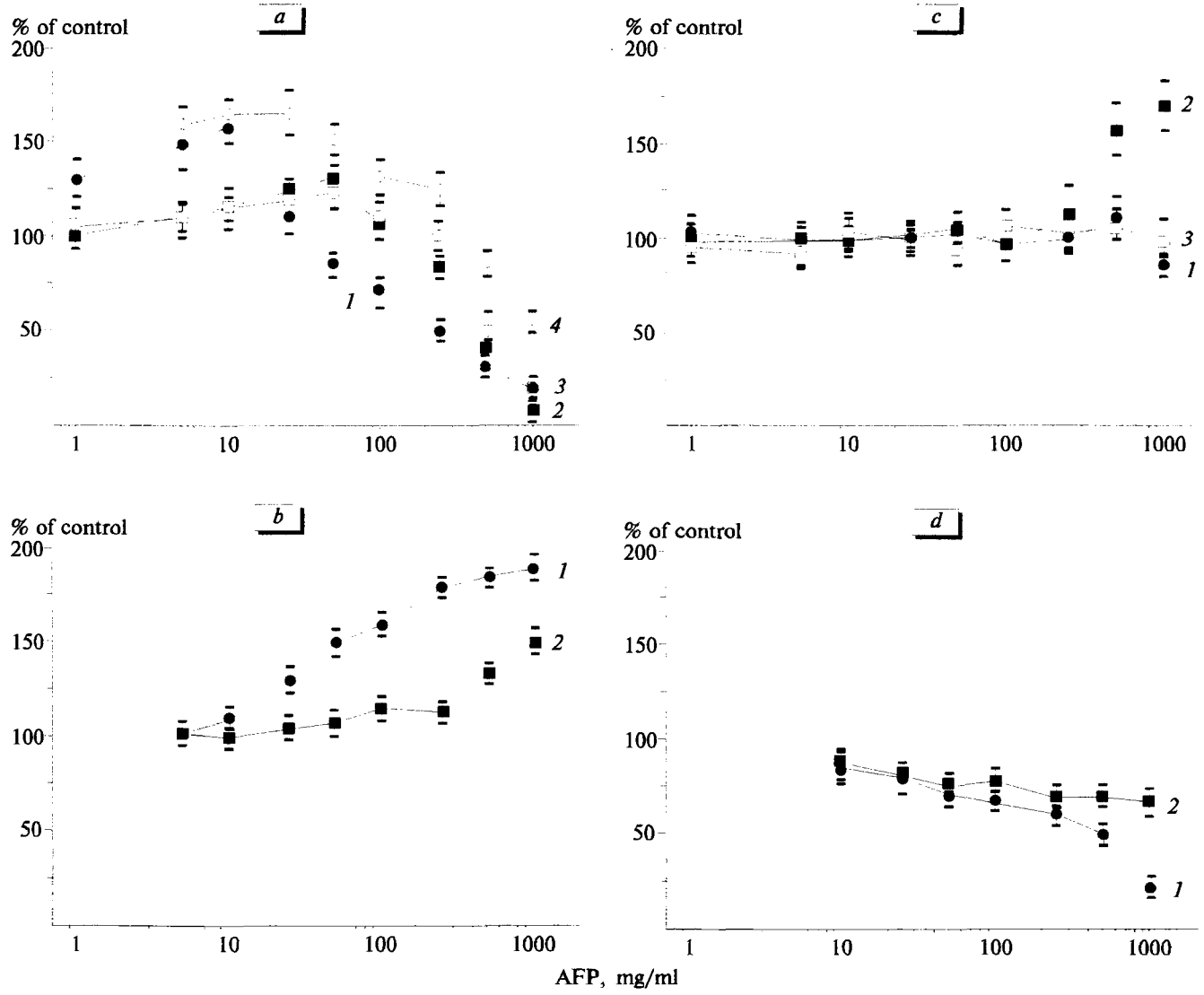

Fig. 1. Effects of embryonic α-fetoprotein (AFP) in various doses on proliferation of tumor cells and normal embryonic fibroblasts: *a*) L-929 (*1*), HepG2 (*2*), MT4 (*3*), and Jurkat cells (*4*); *b*) normal lung fibroblasts lHeF (*1*) and normal retinal fibroblasts rHeF (*2*); *c*) U-937+fetal bovine serum (FBS, *1*), U-937 (*2*), and M19+FBS (*3*); and *d*) MCF-7+FBS (*1*) and MCF-7 (*2*).

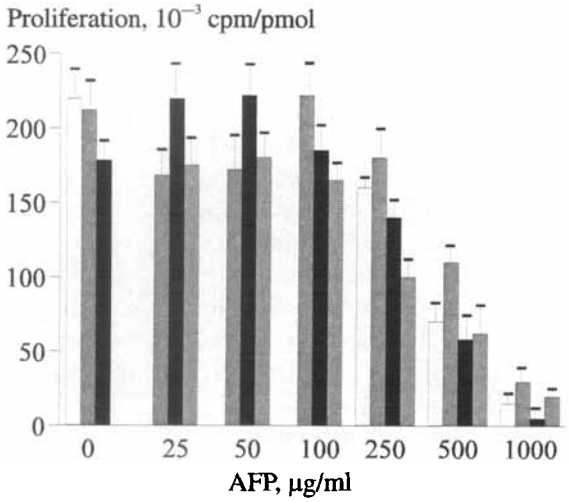

Fig. 2. Effect of 24-h incubation with various α-fetoproteins (AFP) on proliferation of HepG2 cells: embryonic AFP+HSA (1.1 mg/ml, light bars), embryonic AFP+fetal bovine serum (FBS, dark bars), embryonic AFP (shading), cancer AFP (vertical shading), and ligand-free AFP (horizontal shading).

Cytostatic activity of AFP was measured as described elsewhere [5]. The cells (4×10^4/0.15 ml medium) were thoroughly washed with distilled water, placed in a 96-well plate, mixed with AFP or human serum albumin (HSA) in various concentrations, and cultured for 24 h. Cell proliferation was estimated by ^{3}H-thymidine incorporation over the last 4 h of culturing.

Cytostatic activity of AFP was evaluated routinely by trypan blue exclusion.

Signs of apoptosis (*e.g.*, condensation and fragmentation of chromatin, appearance of membrane vesicles, and vacuolization of cell organelles) were evaluated microscopically [34]. The cells treated with AFP in various concentrations were stained with hematoxylin before microscopy. Qualitative electrophoretic analysis of DNA fragments was performed routinely [17,32].

DNA fragmentation in AFP-treated cell was quantitatively analyzed by measuring radioactivity of the cytoplasmic and nuclear fractions in cell lysate after ^{3}H-thymidine labeling [32].

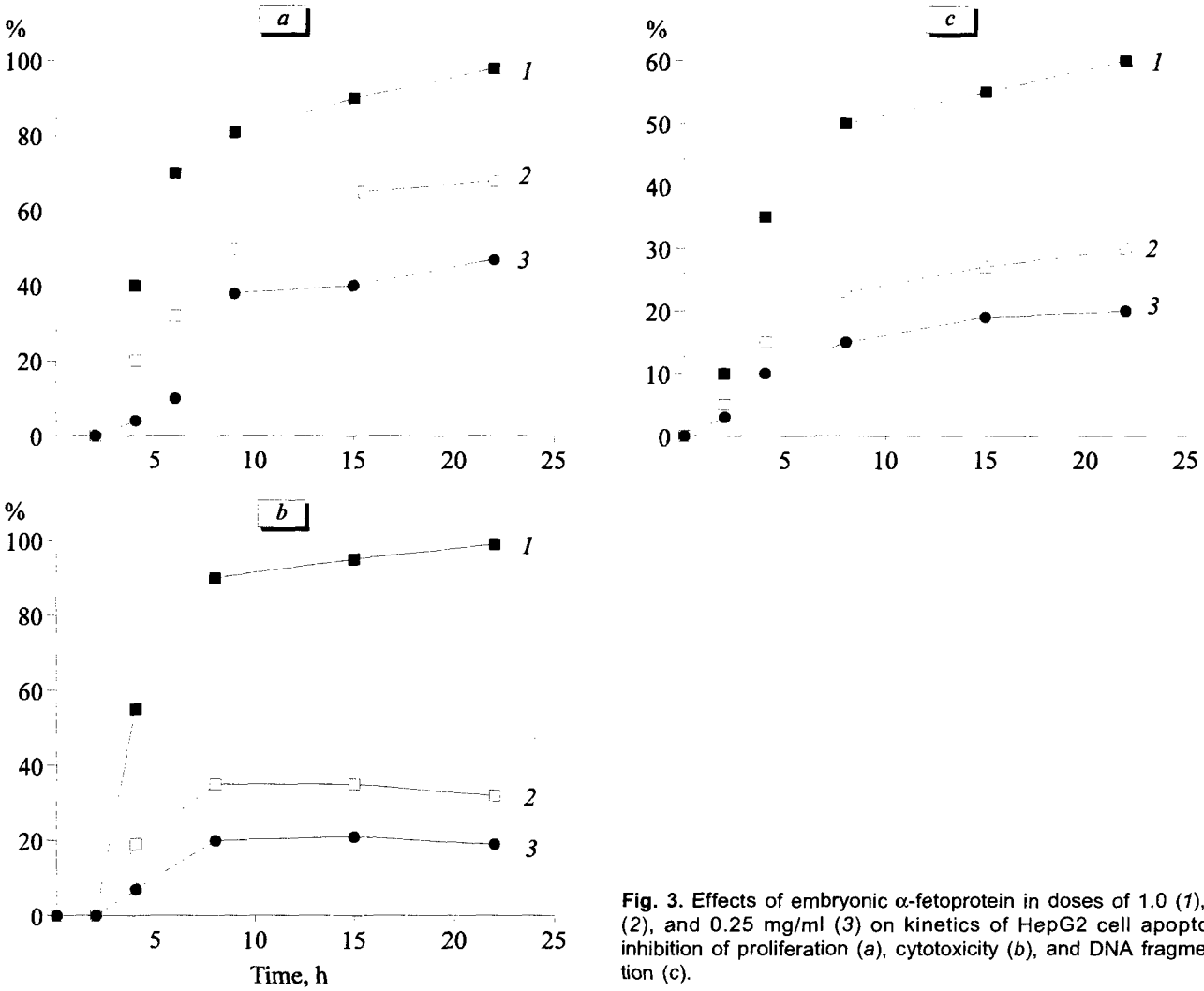

Fig. 3. Effects of embryonic α-fetoprotein in doses of 1.0 (*1*), 0.5 (*2*), and 0.25 mg/ml (*3*) on kinetics of HepG2 cell apoptosis: inhibition of proliferation (*a*), cytotoxicity (*b*), and DNA fragmentation (*c*).

Apoptotic cells were visualized and the effects of AFP on cell cycle were studied by the following method. Nuclei were isolated from cells incubated with AFP, DNA was stained with propidium iodide [4], and the content of DNA was measured on a Becton Dickinson flow fluorescence cytometer equipped with an argon laser at excitation wavelength of 488 nm. The results were analyzed using Lysis II software (Becton Dickinson Immunocytometry Systems). We examined 10^4 cells.

RESULTS

Culturing of HepG2, MT4, Jurkat, and L-929 cells with purified AFP for 24 h produced a dose-dependent effect on DNA synthesis estimated by ^{3}H-thymidine incorporation (Fig. 1, *a*). We constructed curves characterizing the dependence of relative proliferative activity of cells on AFP concentration in the medium. AFP in low doses (below 0.1 mg/ml) acted as a growth factor and stimulated cell proliferation by 20-27% compared to the control. In higher doses AFP inhibited cell proliferation and in the highest dose (1.0 mg/ml) completely suppressed growth of HepG2, MT4, and L-929 cells and caused a 50% inhibition of Jurkat cell proliferation. HSA in the same dose did not inhibit cell proliferation (data not shown).

The effects of AFP on cell growth were also studied in a primary culture of human embryonic fibroblasts. AFP stimulated cell growth by 50-90% compared to the control (similarly to HSA, Fig. 1, *b*).

As differentiated from other cells, U-937 and M19 cells cultured in a medium containing 10% FBS were practically insensitive to AFP. Only in high concentrations (above 0.5 mg/ml) this substance produced cytostatic effects on U-937 cells (30% of the control, Fig. 1, *c*). In a serum-free medium, AFP increased proliferative activity of test cells (Fig. 1, *c*).

Previous studies showed that different sensitivity of cancer cells to cytostatic effects of AFP is associated with the presence of endogenous growth factors and cytokines in the culture medium. Activated Jurkat, HepG2, and U-937 cells secrete interleukin-2, TNF-β, and TNF-α and interleukin-1β, respectively [5]. These cytokines modulate cell sensitivity to AFP. Endogenous and exogenous AFP modulate cell reactions to TNF-α [25]. AFP promotes or blocks cytotoxic effects of TNF-α in suboptimal or high doses, respectively. By contrast, TNF-α suppresses apoptosis caused by AFP in high doses. In addition, endogenous and exogenous interleukin-2 inhibits apoptotic activity of AFP in cultured Jurkat cells [5].

These data suggest that cell response to AFP is a series of processes differently regulated by endogenous cytokines and growth factors.

AFP of different origins are characterized by various carbohydrate compositions and ligand contents [2]. We studied embryonic AFP, cAFP, and ligand-free embryonic AFP. These AFP had similar antiproliferative activity (Fig. 2). AFP in low doses (below 0.1 mg/ml) slightly stimulated cell growth. In higher concentration AFP sharply inhibited or even blocked (1.0 mg/ml) cell proliferation. HSA in a concentration of 1.1 mg/ml did not modulate the effect of AFP. Therefore, these proteins did not compete for binding sites on receptor molecules responsible for transduction of the cytostatic signals. Under conditions of growth factor deficiency (without FBS) AFP in low doses stimulated cell growth, while in high doses this substance markedly suppressed HepG2 cell proliferation.

Thus, cytostatic activity of AFP does not depend on the ligand content, but is determined by the structure and properties of protein macromolecules.

Apoptosis of HepG2 cells was studied at various periods of incubation with AFP (Fig. 3). In our experiments, 24-h incubation with high AFP doses suppressed cell growth and caused their death. Curves characterizing these processes were practically similar ex-

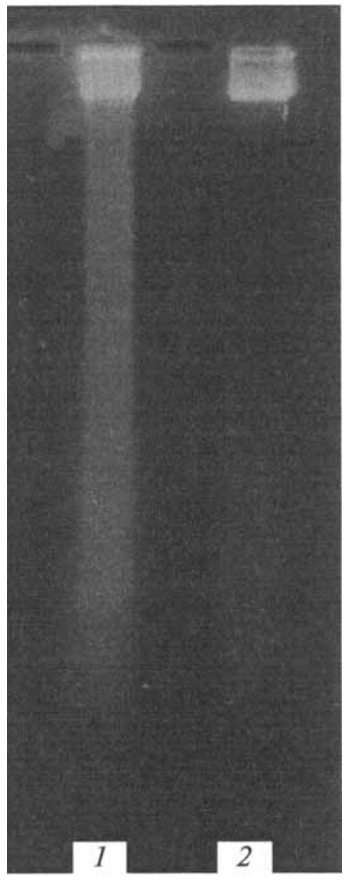

Fig. 4. Agar electrophoresis of DNA from apoptotic HepG2 cells treated with 0.5 mg/ml embryonic α-fetoprotein for 24 h (*1*) and control cells (2).

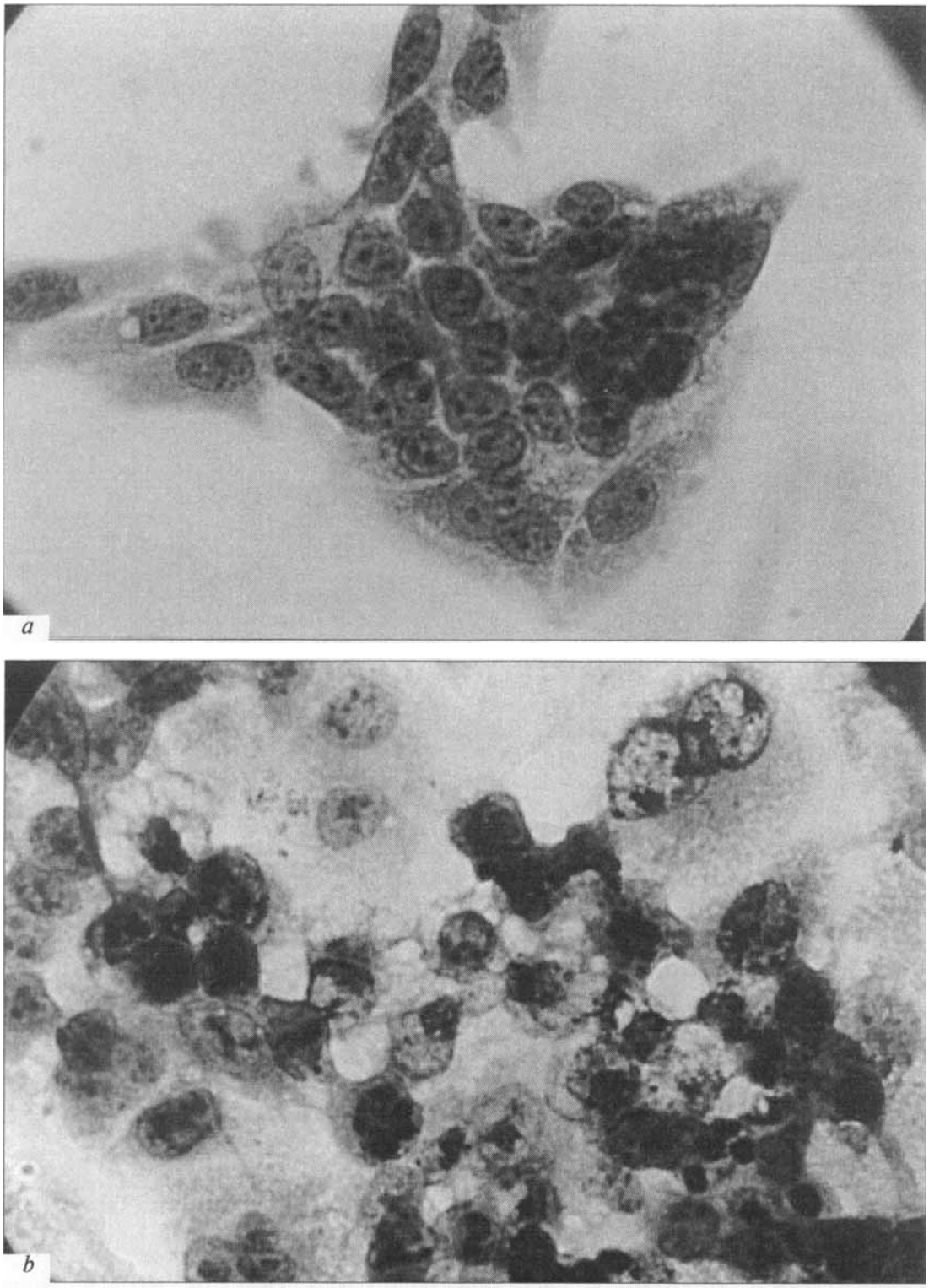

Fig. 5. HepG2 cells incubated in the absence (*a*) and presence of 0.5 mg/ml embryonic α-fetoprotein (*b*) for 24 h. Hematoxylin staining.

cept for the initial portions (Fig. 3, *a*, *b*). DNA fragmentation precedes manifestation of cytotoxicity by several hours. Two-hour incubation of HepG2 cells with AFP produced no cytotoxic effects, while DNA fragments were found in the cytoplasm (Fig. 3, *a*, *c*). Thus, AFP-induced DNA fragmentation precedes other signs of cell death, which is typical of apoptosis [6]. Morphological signs of apoptosis were revealed only after 6-h incubation of HepG2 cells with AFP [24].

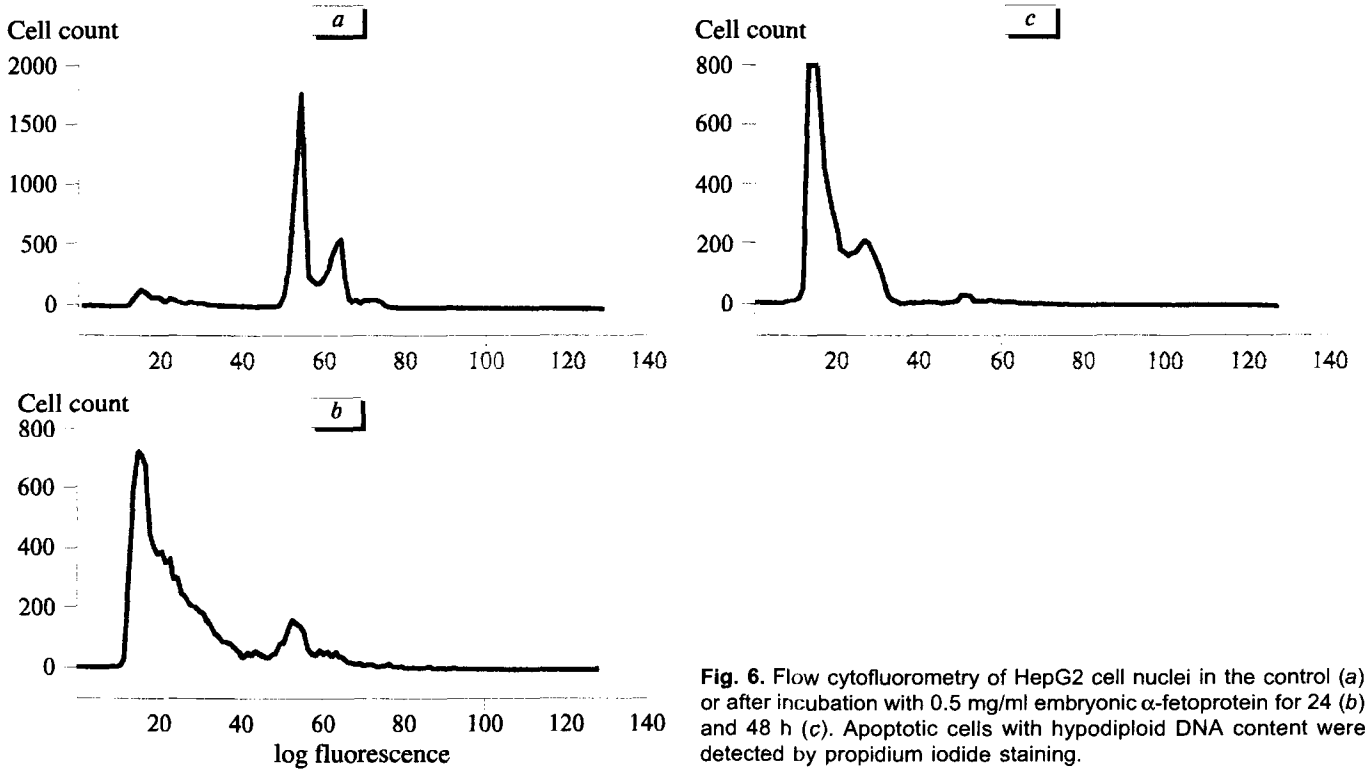

Fig. 6. Flow cytofluorometry of HepG2 cell nuclei in the control (*a*) or after incubation with 0.5 mg/ml embryonic α-fetoprotein for 24 (*b*) and 48 h (*c*). Apoptotic cells with hypodiploid DNA content were detected by propidium iodide staining.

AFP-induced DNA fragmentation was confirmed electrophoretically (Fig. 4). AFP initiated degradation of nuclear DNA into fragments, which appeared as a characteristic apoptotic ladder track on the electrophoretogram.

DNA fragmentation in AFP-treated HepG2 cells was accompanied by the appearance of apoptotic cells (Fig. 5). After 24-h incubation with AFP, signs of apoptosis were found in 50-70% cells (decreased cell volume, condensation and fragmentation of chromatin, vesicles on cell membranes, and vacuolation of cell organelles, Fig. 5, *b*).

In fluorescence histogram of HepG2 cells incubated with AFP, we revealed an additional hypodiploid peak with fluorescence intensity lower than that typical of G_1/G_0 phase cells (Fig. 6, *b*, *c*). This hypodiploid apoptotic peak became more pronounced with increasing the incubation time from 24 to 48 h (Fig. 5, *c*). Decreased DNA content in these cells was related to pronounced chromatin losses during apoptosis. The apoptotic peak was not found on histogram of intact HepG2 cells (Fig. 6, *a*). Computer analysis of spectral histograms showed that 70% nuclei in AFP-treated cells had low chromatin content.

Flow cytofluorometry showed that nuclei in AFP-treated cells were characterized by low light scattering, which indicated their decreased volume and increased density (Fig. 7). It was reported that apoptosis differs from necrosis in decreased volume, but high density of cell nuclei [4].

Thus, human AFP produces a dose-dependent effect (activation or inhibition) on the growth of normal and tumor cells. AFP in low doses stimulates cell proliferation, while in high doses this substance causes pronounced cytostatic and cytotoxic effects on tumor cells.

The effect of AFP on cell growth does not depend on the presence of ligands. At the same time, bioactive ligands enhance or diminish its effects.

The effect of AFP depends on the presence of growth factors and cytokines in the culture medium, which attests to the interrelation between transduction of growth and apoptotic signal pathways. AFP-induced apoptosis is characterized by suppression of cell growth, DNA degradation, and clear morphological signs of programmed cell death.

REFERENCES

1. G. A. Abelev, *Adv. Cancer Res.*, **14**, 295-357 (1971).
2. H. F. Deutsch, *Ibid.*, **56**, 253-312 (1991).
3. H. F. Deutsch, *J. Tumor Marker Oncol.*, **9**, 11-14 (1994).
4. C. Dive, C. D. Gregory, D. J. Phipps, *et al.*, *Biochim. Biophys. Acta.*, **1133**, 275-285 (1992).
5. E. I. Dudich, L. N. Semenkova, E. A. Gorbatova, *et al.*, *Tumor Biol.*, **19**, 30-40 (1998).
6. R. C. Duke, R. Chervenak, and J. J. Cohen, *Proc. Natl. Acad. Sci. USA*, **80**, 6361-6365 (1983).
7. M. Enari, H. Sakahira, H. Yokoyama, *et al.*, *Nature*, **39**, 43-50 (1998).
8. D. Games, D. Adams, R. Alessandrini, *et al.*, *Ibid.*, **373**, 523-527 (1995).

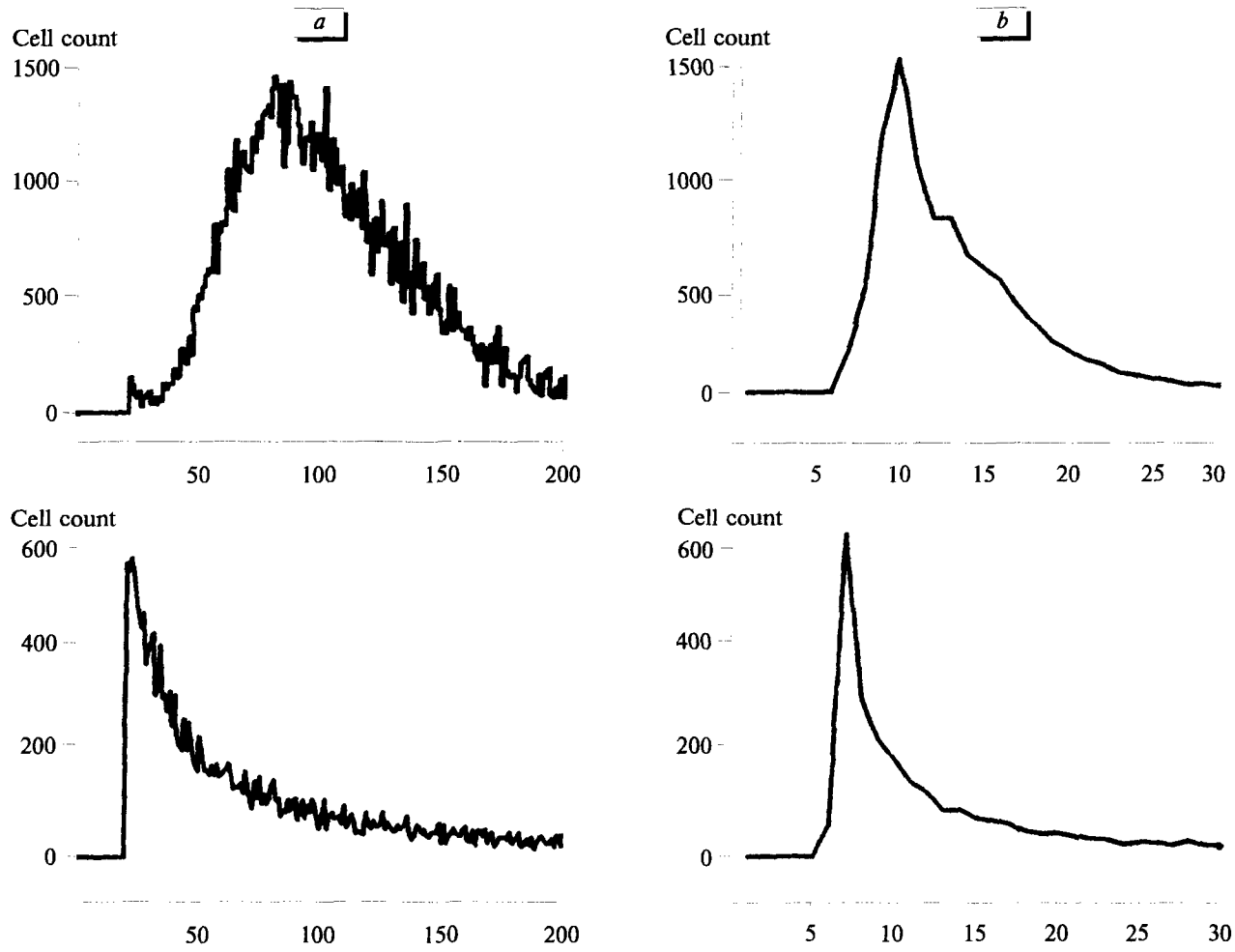

Fig. 7. Flow cytometry of HepG2 cells incubated in the absence (top panels) and presence of 0.5 mg/ml embryonic α-fetoprotein (bottom panels). Straight (*a*) and lateral (*b*) light scattering.

9. D. C. Hooper, B. L. Cohen, D. Ducas, *et al.*, *Biological Activities of alpha-Fetoprotein*, Eds. G. J. Mizejewsky and H. I. Jakobson, Boca Raton (1987), Vol. 1, pp. 153-167.
10. D. W. Hoskin, S. Hamel, D. C. Hooper, and R. A. Murgita, *Cell Immunol.*, **96**, 163-174 (1985).
11. M. Irmler, M. Thome, M. Hahne, *et al.*, *Nature*, **388**, 190-195 (1997).
12. H. I. Jacobson, J. A. Bennett, and G. J. Mizejewski, *Cancer Res.*, **50**, 415-420 (1990).
13. M. D. Jacobson, M. Weil, and M. C. Raff, *Cell*, **88**, 347-354 (1997).
14. B. A. Keel, K. B. Eddy, S. Cho, and J. V. May, *Endocrinology*, **129**, 217-225 (1991).
15. J. Laborda, J. Naval, M. Allouche, *et al.*, *Int. J. Cancer*, **40**, 314-318 (1987).
16. G. J. Mizejewsky, J. F. Keenan, and R. P. Setty, *Biol. Reprod.*, **52**, 887-898 (1990).
17. R. J. Mogil, Y. Shi, R. P. Bissonette, *et al.*, *J. Immunol.*, **152**, 1674-1683 (1994).
18. S. Nagata, *Cell*, **88**, 355-365 (1997).
19. J. Naval, M. Villicampa, A. Goguel, and J. Uriel, *Proc. Natl. Acad. Sci. USA*, **82**, 3301-3305 (1985).
20. S. Nishi and H. Hirai, *Biochim. Biophys. Acta*, **278**, 293-298 (1972).
21. E. A. Nunez, *Tumor Biol.*, **15**, 63-72 (1994).
22. D. Parmelee, M. A. Evenson, and H. F. Deutsch, *J. Biol. Chem.*, **253**, 2114-2119 (1983).
23. D. J. Semeniuk, R. Boismenn, and J. Tam, *Adv. Exp. Med. Biol.*, **383**, 255-269 (1995).
24. L. N. Semenkova, E. I. Dudich, and I. V. Dudich, *Tumor Biol.*, **18**, 261-274 (1997).
25. L. N. Semenkova, E. I. Dudich, I. V. Dudich, *et al.*, *Ibid*, **18**, 30-40 (1997).
26. C. Sonnerschein, A. A. Ucci, and A. M. Soto, *J. Natl. Cancer. Inst.*, **64**, 1147-1152 (1980).
27. C. Terai, R. S. Kornbluth, C. D. Pauza, *et al.*, *J. Clin. Invest.*, **87**, 1710-1725 (1991).
28. M. Thome, P. Schneider, K. Hofmann, *et al.*, *Nature*, **386**, 517-521 (1997).
29. C. B. Thompson, *Science*, **267**, 1456-1462 (1995).
30. D. L. Vaux and A. Strasser, *Proc. Natl. Acad. Sci. USA*, **93**, 2239-2244 (1996).
31. D. Wallach, *Nature*, **388**, 123-126 (1997).
32. A. H. Wyllie, *Ibid.*, **284**, 555-556 (1980).
33. A. Wyllie, *Ibid.*, **391**, 20-21 (1998).
34. A. H. Wyllie, J. F. R. Kerr, and A. R. Currie, *Int. Rev. Cytol.*, **68**, 251-306 (1980).
35. T. Yamashita, A. Nakane, and T. Watanabe, *Biochem. Biophys. Res. Commun.*, **201**, 1154-1159 (1994).