Flow cytometry and biochemical analysis of DNA degradation characteristic of two types of cell death

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By flow cytometry it has been shown that in rat thymocytes dying upon γ-irradiation the reduction in DNA content per cell occurs well before the increase in outer membrane permeability. In contrast, in irradiated Burkitt's lymphoma cells the disturbance of plasma membrane permeability precedes the decrease of DNA content. Genome degradation in dying thymocytes of irradiated or hydrocortisone-treated rats is accounted for by internucleosomal DNA fragmentation. Postirradiation DNA cleavage in the 3 investigated cell cultures is disorderly and is probably caused by the activation of hydrolases in dead cells.

Cell death *DNA degradation* *Flow cytometry* *Membrane permeability*

1. INTRODUCTION

It has been shown for various systems that the death of eucaryotic cells is accompanied by fragmentation of nuclear DNA [1–3]. Genome degradation is often the first irreversible event in a dying cell. The early DNA cleavage is characteristic of programmed cell death in embryogenesis [4], of lymphoid cell death induced by irradiation [5–7], glucocorticoids [7,8] or alkylating agents [9], of cytolysis induced by T-killers [10], etc. In all these cases chromatin degradation is accounted for by the ordered internucleosomal fragmentation of nuclear DNA preceding the disturbance of cell membrane permeability. Similar genome degradation is also observed in the course of terminal differentiation of some cells (mammalian erythrocytes, lens cells), resulting in nucleus elimination [11].

By morphological criteria, 2 types of cell death are distinguished: (i) apoptosis which begins with a shrinkage of the nucleus and the whole cell; (ii) necrosis which is conversely characterized by the swelling of organelles and the whole cell, resulting in the rupture of plasma membrane. In this case the changes in the nucleus are rather late and seem to be secondary [12]. The early ordered internucleosomal fragmentation of chromatin is peculiar to apoptosis. The state of the genome at necrotic cell death has not yet been studied.

Flow cytometry of cells, stained with DNA-specific probes, was recently shown to be informative in the analysis of DNA degradation and its loss from dying cells [13]. These processes are manifested as the appearance of cells with DNA content lower than 2C on histograms.

The aim of this work was to study the kinetics and character of DNA degradation as well as the disturbance of plasma membrane permeability in the 2 types of cell death. Thymocytes of irradiated or glucocorticoid-treated rats were used as cells dying by apoptosis. Dividing cells usually undergo necrosis upon γ-irradiation. And so irradiated cells of Burkitt's lymphoma (strain Raji), fibroblasts of Chinese hamster (strain B11-d-ii-FAF 28) and BHK-21 Syrian hamster cells were investigated as potentially necrotic.

2. MATERIALS AND METHODS

Male Wistar rats of 120–150 g body wt and the above-mentioned cell cultures were used in the ex-

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periments. Rats and cultivated cells were irradiated with ^{137}Cs γ-rays at a dose of 10 Gy. Hydrocortisone acetate was introduced at a dose of 10 mg per 100 g body wt. To obtain a thymocyte suspension, thymus was removed and rubbed through a nylon net.

2.1. *Estimation of the outer cell membrane permeability*

The cells were suspended in Hanks' medium, where phenol red was substituted by ethidium bromide at 10 μg/ml, and 2 min later placed in a chamber of a cytofluorometer. In these experiments we used a special chamber with a strictly constant flow rate of cell suspension. First, the average number of fluorescent cells, with initially high permeability for ethidium bromide, passing through the measuring chamber per unit time was determined. Second, digitonin (40 μg/ml) was added to make all the cells permeable for ethidium bromide and the measurement was repeated. The percentage of permeable cells was calculated by dividing the values obtained before and after digitonin addition. Fluorescence of ethidium bromide was excited by laser-line 488 nm and registered at 600 nm.

2.2. *DNA flow cytometry*

The cells were sedimented by 10 min centrifugation at 300 × *g*, suspended in 0.14 M NaCl, 0.01 M Tris-HCl, pH 7.2, and fixed by 50% ethanol for 1.5 h at −18°C. 90 μl fixed cells (≈45 × 10^7 cells/ml) were added to 3 ml of solution containing 12.5 μg/ml ethidium bromide, 25 μg/ml mithramycin, 3.75 μM $MgCl_2$, 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.4. The cells were stained for 30 min and analysed on a flow cytofluorometer. Mithramycin was excited at 405 nm and the fluorescence of ethidium bromide was registered at 600 nm. As a result of energy transfer, the excitation of mithramycin, specifically bound to GC pairs of DNA, led to ethidium bromide fluorescence, the intensity of which is proportional to the DNA content in individual cells [13,14].

Data were analysed by using a nonparametric method of histogram treatment.

2.3. *Isolation and electrophoretic analysis of DNA*

DNA isolation and electrophoresis in 3% polyacrylamide gel–0.5% agarose were performed as described [7].

3. RESULTS AND DISCUSSION

DNA histograms of rat thymocytes and Burkitt's lymphoma cells are reproduced in fig.1. A considerable amount of thymocytes with <2C DNA content appears by the 6th hour after irradia-

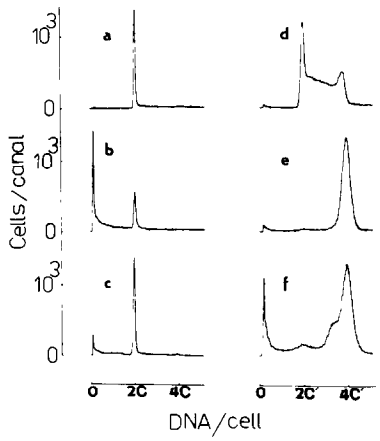

Fig.1. DNA histograms of thymocytes (a–c) and Burkitt's lymphoma cells (d–f). (a,d) Controls; (c) 6 h after hydrocortisone injection; (b,e,f) 6, 24, 48 h after irradiation, respectively.

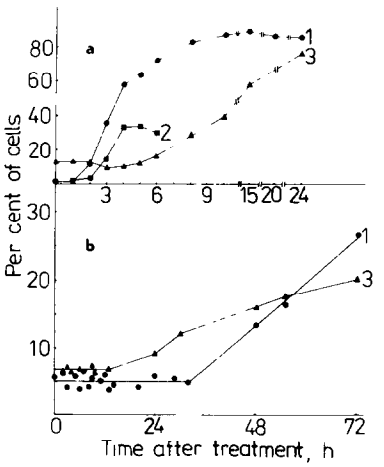

Fig.2. Kinetics of appearance of cells with <2C DNA content (1,2) and those permeable for EtBr (3). (a) Thymocytes, (b) Burkitt's lymphoma cells. (1,3) Irradiation, (2) hydrocortisone injection. Each point is the average of 4–6 independent experiments.

tion or hydrocortisone injection (fig.1b,c). In the irradiated culture such cells are observed after the 24th hour only (fig.1f). The synchronization of lymphoma cells in the G_2 period (fig.1) is explained by an almost complete block of mitosis.

In fig.2 the kinetics of accumulation of cells with <2C DNA and those permeable for ethidium bromide are compared. In dying thymocytes chromatin degradation and its loss from cells begins after a 1–2 h lag period and reaches maximal values 8–10 h after irradiation or 4 h after hydrocortisone treatment. In the last case the amount of <2C DNA cells corresponds to the portion of thymocytes sensitive to hormone by a cytolytic test. The permeability of the thymocyte outer membrane remains practically unchanged throughout the 6 h period after irradiation. In contrast, in irradiated Burkitt's lymphoma the appearance of <2C DNA cells is preceded by an increase in the number of cells permeable for ethidium bromide.

It should be noted that, due to high background fluorescence in a chamber used for analysis of membrane permeability, weakly fluorescent cells with low DNA content are not registered. This leads to the underestimation of permeable cells by the later time after irradiation.

The subsequent experiments showed that not only the sequence of events but also the character of DNA degradation are different for the 2 types of cell death. At various times after cell treatments, DNA was isolated and subjected to electrophoresis. Fig.3a,b shows that ordered internucleosomal DNA cleavage occurs in thymocytes dying after irradiation or hydrocortisone injection. On the other hand, we failed to find nucleosomes and their oligomers in the irradiated cell cultures up to the 96th hour after irradiation. A small amount of DNA fragments observed are irregular, which indicates the simultaneous attack of chromatin by nucleases and proteases. Control experiments showed that DNA fragments in the culture medium were lacking.

Thus, the flow cytometry and biochemical studies performed reveal 2 forms of DNA degradation, characteristic of 2 types of cell death:

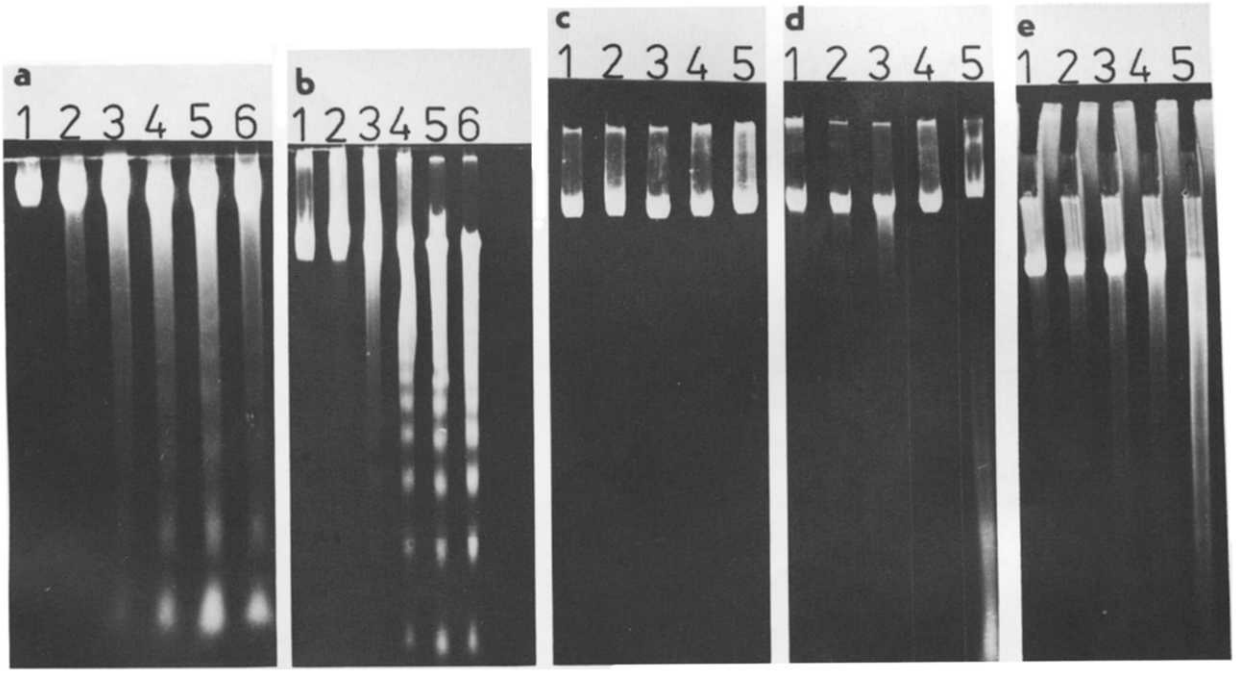

Fig.3. Electropherograms of DNA, isolated from rat thymocytes (a,b), Burkitt's lymphoma (c), Chinese hamster fibroblasts (d) and BHK cells (e). Lanes: 1, control; (a) 2–6, 1, 2, 4, 6, 8 h after irradiation; (b) 2–6, 1, 2, 3, 4, 6 h after hydrocortisone injection; (c–e) 2–5, 24, 48, 72, 96 h after irradiation.

(i) Early internucleosomal chromatin fragmentation, which precedes the disturbance of outer cell membrane permeability, is characteristic of that type of cell death which is morphologically defined as apoptosis. The regular character of DNA cleavage, its dependence on protein synthesis and energetic processes, the possibility to prevent cell death by inhibitors of Ca^{2+},Mg^{2+}-dependent nuclease and a number of other data make it possible to suppose that this type of cell death is related to the realization of a specific genetic program [2,3,10,12].

(ii) The necrotic type of cell death is characterized by an early increase in plasma membrane permeability with a subsequent irregular DNA degradation, which is probably caused by the activation of hydrolases in dead cells.

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