

Internucleosomal chromatin degradation in myeloma and B-hybridoma cell cultures

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The activity of Ca/Mg-dependent endonuclease (CME) is strongly inhibited in myeloma X-63.Ag8.653 and B-hybridoma MLC-1c as compared with mouse splenocytes. Nevertheless, pronounced internucleosomal chromatin degradation occurs in both cell lines during long-term cultivation without passing. In isolated cell nuclei of X-63 the activation of CME, which precedes chromatin fragmentation *in vivo* and loss of cell viability, is revealed. The time-course of CME activation is opposite to cell proliferation and is not accompanied by alterations in enzyme quantity. The results suggest that cell death of X-63 and MLC-1c occurs via apoptosis, and involves the mechanisms controlling the activation and/or interaction of CME with chromatin.

Hybridoma; Apoptosis; Ca/Mg-dependent endonuclease

1. INTRODUCTION

Apoptosis, or programmed cell death, has been widely discussed in the last few years [1]; it seems to be a common form of cell death during normal development and a cellular response to various factors [2,3]. The biochemical hall-mark of apoptosis is internucleosomal chromatin fragmentation leading to a specific ladder of mononucleosomes and their oligomers [4]. This process has been described for various types of lymphocytes, hemopoietic precursors and T-hybridomas, and has been suggested for the activation of nuclear endonuclease(s) and/or alteration of their interactions with chromatin [5–7]. In this paper we show that, in myeloma X-63 and B-hybridoma MLC-1c cells, internucleosomal chromatin degradation occurs during maintenance without medium replacement. In X-63 this process is accompanied by activation of chromatin cleavage in cell nuclei by Ca/Mg-dependent endonuclease (CME), but not by acid DNase. The amount of enzyme in cell nuclei remains constant during myeloma maintenance. It is believed that mechanisms controlling CME interaction with chromatin may be the critical point in apoptotic induction.

2. MATERIALS AND METHODS

2.1. Cell lines and animals

Mouse myeloma X-63, Ag8.653 and hybridoma MLC-1c cells were

grown in Dulbecco's modified Eagle's medium containing 15% bovine serum albumin (BSA), 44 mM sodium bicarbonate, 25 mM HEPES and 100 mg/ml gentamicin. Cells were seeded at a concentration of 10^6 cells/ml and were kept at 37°C in 5% CO₂/95% air incubator for 12 days without passing. MLC-1c was derived by fusion of X-63 and Balb/c mice splenocytes immunized by light chain I of human myosin. Cell viability was measured by Trypan blue exclusion. Proliferation assays were performed as described in [8]. Balb/c mice were used in experiments for isolation of splenocyte cell nuclei.

2.2. Activation and measurement of endonucleases in cell nuclei

Isolation of cell nuclei was performed as described in [9] with slight modifications. The nuclei were transferred in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM CaCl₂, 0.25 M sucrose for activation of CME, or 50 mM sodium acetate, pH 4.8, 1 mM Na₂EDTA for activation of acid DNase, and incubated for 1 h at 37°C. Nuclei were lysed in 1 M NaCl, 0.5% SDS, incubated with RNase A (200 mg/ml) for 1 h at 37°C, proteinase K (50 mg/ml) for 1 h at 37°C, and twice deproteinized with chloroform/isoamyl alcohol (24:1). DNA was separated electrophoretically in 1.2% agarose gels. DNA was visualized by staining with ethidium bromide, photographed in UV light and negatives were scanned on an Ultrosan XL densitometer. The nuclear endonuclease activity was measured according to [10].

2.3. Immunohistochemical analysis of CME in isolated cell nuclei

ELISA was carried out with the monoclonal antibody, DN [11]. 96-well microplates were coated with nuclear extract (10 mg/ml) prepared as described in [11], incubated with 1% BSA, washed and incubated with antibody DN diluted in 0.01 M phosphate buffer, pH 7.5 containing 0.015 M NaCl (1:1,000), then with biotinylated goat anti-mouse antibodies followed by streptavidin-biotinylated horse-radish peroxidase complex (Amersham, UK), *o*-Phenylenediamine-H₂O₂ was used as the peroxidase substrate.

3. RESULTS

Fig. 1 demonstrates the comparative activity of CME in isolated nuclei of mouse splenocytes and 3-day cul-

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tures of myeloma and hybridoma cells. CME is easily activated in splenocyte cell nuclei, leading to rapid internucleosomal chromatin degradation. In contrast, only restricted CME activity and no demonstrable pronounced internucleosomal chromatin degradation, even after 2 h incubation, are found in myeloma and hybridoma cell nuclei.

We studied the possibility of CME activation and endonuclease(s)-driven chromatin degradation *in vivo* and *in vitro* during long (up to 10–12 days) maintenance of cell lines without medium replacement. The results are shown in Figs. 2 and 3. Both cell lines grow in these conditions up to the 7–8th day. During the first 6 days the cell populations maintain their initial viability, but from the 7th day a rapid decrease in viability occurs. Fig. 3 shows that loss of viability is accompanied by chromatin cleavage *in vivo* in both cell lines. In MLC-1c the pronounced sign of that process is revealed on the 6th day of cultivation, and from the 7th day the typical nucleosomal ladder is observed. In X-63 the bulk of the nuclear DNA converted to nucleosomes and their oligomers, also on the 7th day of cultivation, and a different character of DNA cleavage in native and incubated myeloma nuclei, was found. We may conclude that under the conditions used, the loss of viability of myeloma and hybridoma cells is connected with internucleosomal chromatin degradation. We examined the activation of CME in isolated cell nuclei of both cell lines in the time-course of their cultivation. No enzyme activation was observed in isolated nuclei of B-hybridoma MLC-1c cells, even though there was pronounced DNA degradation *in vivo* (Fig. 3B). We were unable to detect enzymatic activity in isolated cell nuclei of hybridoma cells, although using the monoclonal antibody, DN, to CME we detected the specific protein in extracts, and no significant change of CME content was found.

The pronounced increase in enzyme activity is observed in X-63 from the 4th day (Figs. 3A and 2C). Up to the 6th day it reaches an almost maximum level, while only from that day onwards is the decrease in cell viability observed (Fig. 2B). So, in myeloma cells, CME activation precedes cell death and detectable DNA fragmentation *in vivo*. Immunochemical estimation of the CME amount in extracts did not reveal parallel changes in the specific protein content (Fig. 2C).

As can be seen in Fig. 2D, a drastic decrease in proliferative activity in X-63 occurs beginning from the 4th day. It is interesting that the time-course of relative proliferative activity (defined as the total proliferation divided by the amount of viable cells) is equal to the time-course of total proliferation. This indicates that there is a uniform decrease in proliferation activity of viable cells in the culture. It is known that some types of acid DNAses can exist in cell nuclei [12]. We measured CME and acid DNase activity in isolated nuclei of X-63 from 5-day cultures. The activity of acid DNase was very low (5 U) as compared with CME

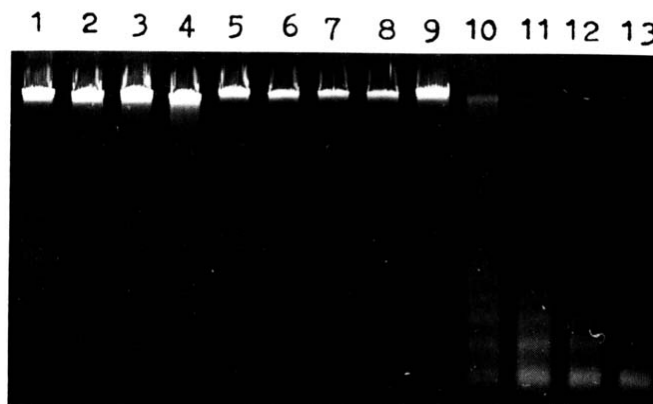


Fig. 1. CME activation in isolated cell nuclei of myeloma X-63, Ag8.653 (lanes 1–4), hybridoma MLC-1c (lanes 5–8) and Balb/c splenocytes (lanes 9–13). Cells were harvested on the third day of cultivation. Time of incubation: 15 min, lane 10; 30 min, lanes 2,6,11; 60 min, lanes 3,7,12; 120 min, lanes 4,8,13. Control, lanes 1,5,9.

activity (43 U). Even with longer incubations of myeloma nuclei in buffer medium for acid DNase we did not observe internucleosomal fragmentation of chromatin.

4. DISCUSSION

The obtained results show that CME activity in nuclei of myeloma X-63 cells is not detectable, the opposite situation to that observed in Balb/c splenocyte nuclei in which CME is easily activated and cleaves chromatin DNA. In MLC-1c, which is a hybrid of X-63 and splenocytes, the enzyme activity and its interaction with chromatin are similar to the myeloma cells and not to the splenocytes. So the tumor, hybrid cells demonstrate restricted CME activity and/or its interaction with chromatin in isolated cell nuclei as compared with splenocytes.

Long-term cultivation of both hybridoma and myeloma cell lines leads to the internucleosomal fragmentation, which is one of the main biochemical signs of apoptosis or programmed cell death [4,6]. The induction of apoptosis can be achieved by the appearance or disappearance of necessary factors, for example different interleukins [1,6], but the secretion of some specific factor(s) during long-term cultivation of X-63 and MLC-1c cannot be excluded. Analogous results were obtained for B-hybridoma cells set up in protein-free medium [13].

CME activation in X-63 cell nuclei precedes internucleosomal chromatin degradation in cultivated cells. This result is similar to some reported cases of CME activation as one of the early events of cell death [4,14]. It should be noted that the time-course of CME activation is opposite to that of proliferative activity. Probably, the mechanisms which stimulate cell proliferation lead to inhibition of CME and/or restriction of its inter-

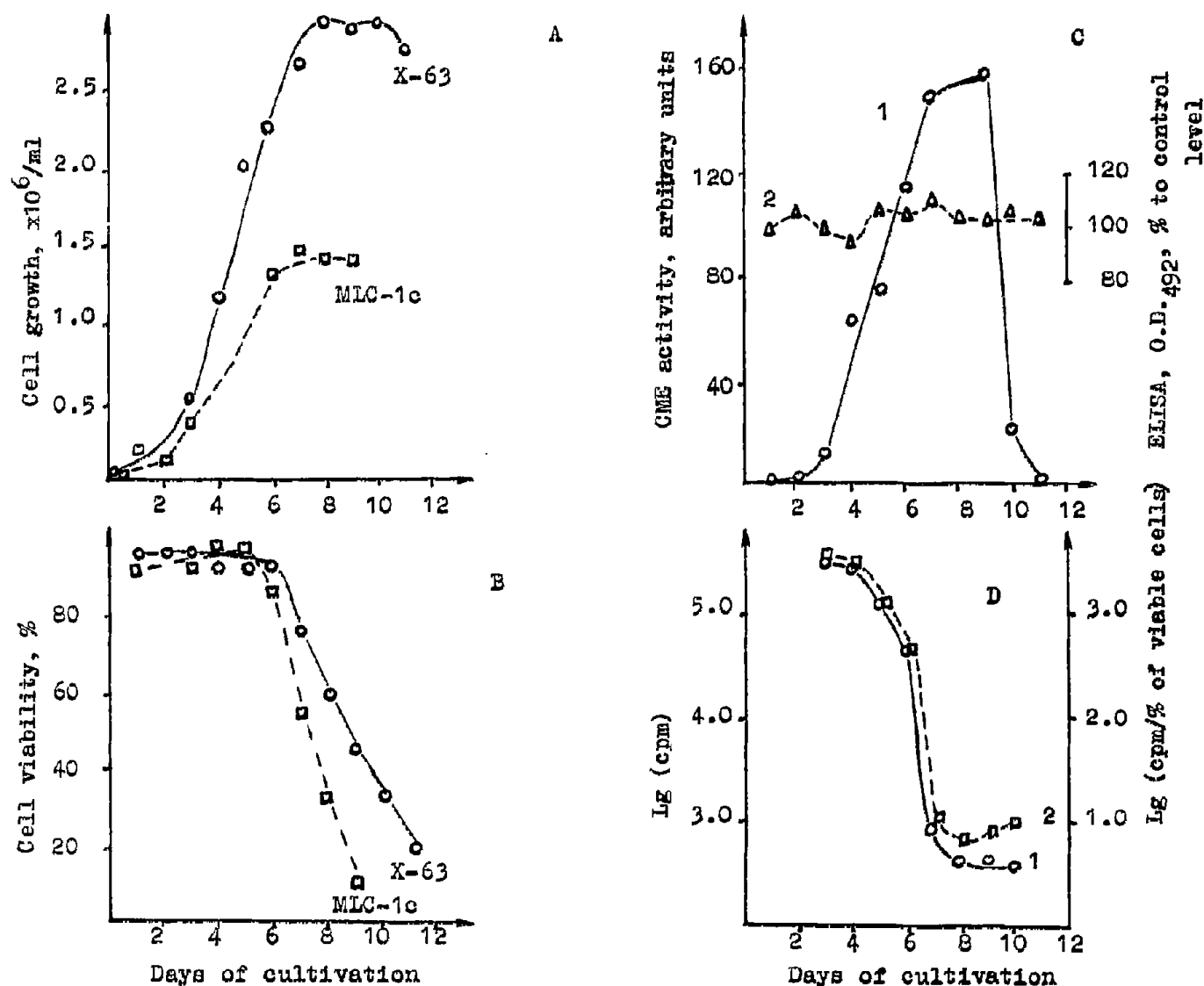


Fig. 2. Characteristics of cell growth, proliferation and CME changes during cultivation of myeloma X-63.Ag8.653 and hybridoma MLC-1c. (A) Cell growth, (B) cell viability and (C) CME changes during cultivation of X-63 (line 1, $\circ$, enzyme activity in isolated cell nuclei; line 2, Δ enzyme content in nuclear extracts by ELISA with monoclonal antibody DN according to section 2. (D) Proliferation activity of X63 line 1, $\circ$, total ^3H incorporation; line 2, $\square$, relative ^3H incorporation (see section 2).

actions with chromatin [7,15]. We observed a similar situation in nuclei of peripheral blood lymphocytes during chronic lympholeucosis [16]; in this case the specific protein inhibitor was revealed. The results suggest that, in the cells studied, the regulation of the enzyme is not achieved through de novo CME synthesis and/or its transport into the nuclei, but realized through another mechanism [17].

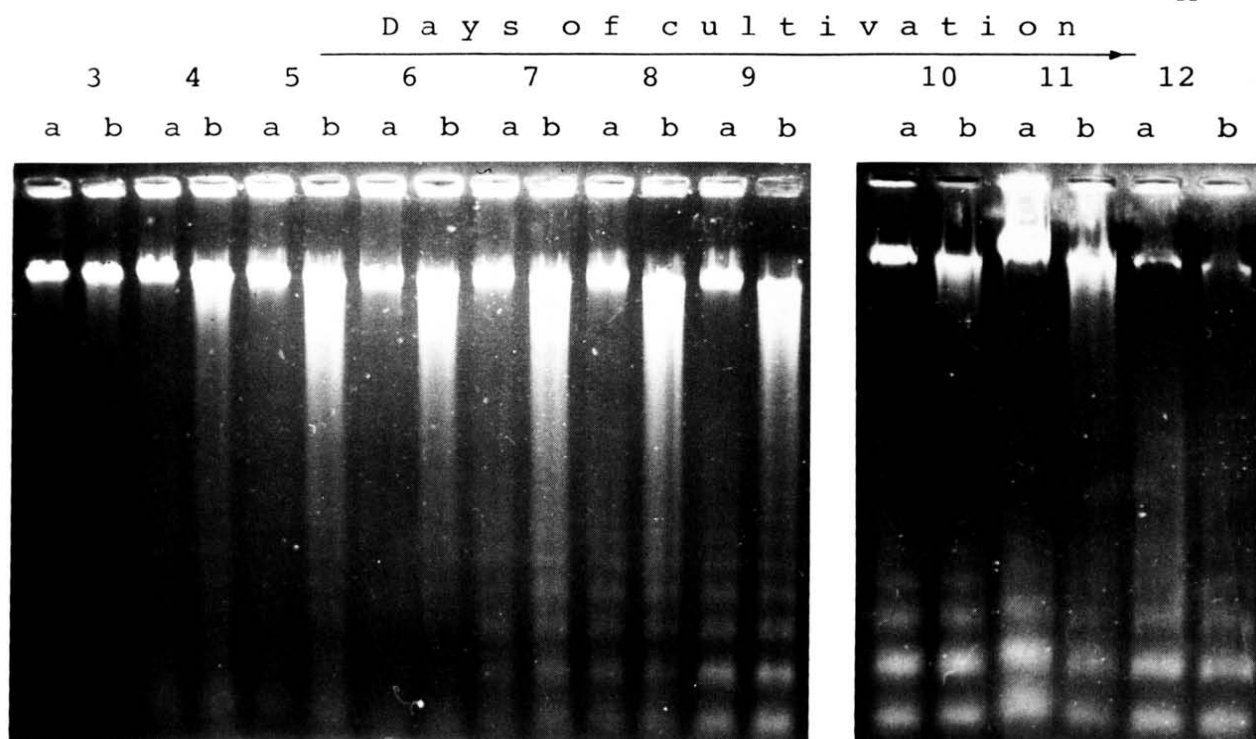
During the later stages of cultivation deep fragmentation of nuclear DNA is achieved both in X-63 and in MLC-1c when most of the chromatin is converted to mononucleosomes (Fig. 3). This would be possible only when CME is presented in the nuclei in the free form (not bound to chromatin) and every linker segment is accessible to the enzyme. However, biochemical evi-

dence has shown that the enzyme is bound to chromatin and can only be eluted with salt [18].

A lot of information devoted to the high order structure of chromatin, which determines non-random accessibility of its different regions to endonucleases, has accumulated [19]. This finds that both factors must undergo alterations during the early steps of apoptotic induction — the enzyme fixation in chromatin and the chromatin structure itself. These processes seems to be critical points in understanding the role of CME and other endonucleases in apoptotic induction, and are the aims of our further investigations.

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A



B



Fig. 3. Chromatin degradation in vivo and in vitro during cultivation of X-63 (A) and MLC-1c (A). For each day of cultivation a means DNA extracted from native isolated nuclei and b means DNA from isolated nuclei, incubated for 1 h in buffer medium for CME activation (see [8]). Arrows indicate the position of molecular size markers (*Hind*III digest of λ -DNA).

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