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EFFECTS OF SERUM AND PROTEIN SYNTHESIS INHIBITORS ON POLYMORPHONUCLEAR NEUTROPHIL APOPTOSIS

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ABSTRACT

In this study the programmed cell death (apoptosis) of human neutrophilic granulocytes was investigated in the presence and absence of fetal calf serum (FCS), cycloheximide and actinomycin-D. The results show that when FCS is omitted from cultures, apart from a decrease in viability, the percentage of apoptotic cells and DNA fragmentation increases. Apoptosis is accelerated in serum withdrawal cultures at 6 hours of incubation time. The use of fluorescent dyes and diphenylamine reaction procedures confirm the above results. Treatment of cells with protein synthesis inhibitors, actinomycin-D and cycloheximide promotes apoptosis and produces a typical ladder of internucleosomal cleavage in the cellular chromatin; the extent of fragmentation however, differs.

Keywords: Apoptosis, polymorphonuclear neutrophil, cycloheximide, actinomycin D.

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INTRODUCTION

Cells can be harmed by a wide range of pathological stimuli, such as certain toxins or viruses. Defective cells die by a process known as necrosis, which involves the disruption of membrane integrity and subsequent cellular swelling, resulting in cell lysis.[1] In contrast, programmed cell death or apoptosis is a normal physiological process. Morphological studies have shown that cells which die by physiological mechanisms often undergo apoptosis.[2-4] The external signals that lead to apoptosis are probably as varied as those that lead to differentiation and proliferation and may include the presence or withdrawal of extracellular signals.[5-7]

Human neutrophils undergo senility and aged neutrophils are recognized and ingested by macrophages.[8] In recent decades, it has become apparent that inflammation is also implicated in the pathogenesis of many diseases that have assumed prominence in developed societies.[9] Polymorphonuclear neutrophils (PMNs) play a central role in inflammation.[10] During the inflammatory response, PMNs leave the circulation on appropriate stimulation and enter the inflamed area to exert their biological function.[11] Various cytokines released during inflammation in an autocrine or paracrine manner may then regulate the survival of PMNs in the lesion either by promoting or by inhibiting their death.[12] It has been recently shown that aging PMNs undergo characteristic changes indicative of apoptosis, including cell shrinkage, nuclear chromatin condensation and DNA fragmentation into nucleosome length fragments.[13-15] The process of programmed cell death (PCD) may then allow recognition and ingestion of apoptotic cells by macrophages and thus may represent a mechanism by which PMNs are cleared from the inflammatory site.[10,15] In this report we have examined the influence of fetal celf serum (FCS) and the effect of actinomycin D and cycloheximide on PMN apoptosis.

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MATERIAL AND METHODS

All reagents were of analytical grade: proteinase K, EcoR1(DNA standard), Ficoll histopaque-1077, dextran type-580, cycloheximide, actinomycin D, trypan blue, ethidium bromide, and acridine orange were purchased from Sigma Chemical Co. Dulbecco's Modified Eagle Medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco. Diphenylamine and other chemical compounds were obtained from Merck.

Cell purification

Human polymorphonuclear neutrophils from the peripheral blood of healthy volunteers were prepared by the modified method of Böyum.[16] Briefly, dextran-580 was added to fresh venous blood containing EDTA as an anticoagulant and sedimented for 1.5 hours at room temperature and the leukocyte-rich plasma was then centrifuged for 10 min. at 875 g. The resulting leukocyte pellet was resuspended in 8 mL of plasma and centrifuged on 3 mL Ficoll for 30 min. at 573 g. The supernatant including the interface of mononuclear cells was discarded carefully and the residual red cells existing in the PMN pellet were lysed in 1mL ammonium chloride solution containing 0.16 M ammonium chloride (NH_4Cl), 10 mM $KHCO_3$, 0.1 mM EDTA, and 10 mL DH_2O, pH= 7.2 and centrifuged for 30 seconds at 2000 g. The pellet was then resuspended in 1mL DMEM supplemented with 200 U/mL penicillin and 200 µg/mL streptomycin. PMN viability was assessed by the ability to exclude trypan blue and cell survival was expressed as the percentage of control cells surviving at hour zero. Isolated cells were 98% pure PMNs as assessed by microscopic examination of slide preparations fixed in methanol and stained with Wright-Geimsa stain.

Cell culture

Freshly isolated cells were suspended at a density of 5×10^6 cells/mL in DMEM with or without 20% fetal calf serum (FCS) and incubated for various periods of time in tissue culture tubes at 37°C in a humidified atmosphere of 5% CO_2 in air. In another experiment, the culture was carried out in the presence or absence of 20 µg/mL actinomycin D or cycloheximide.

Apoptotic index

Apoptotic cells were determined by using a mixture of acridine orange and ethidium bromide of 0.4 µg/100 mL final concentration. 300 cells were counted and recorded as normal versus apoptotic nuclei. The apoptotic index and the percentage of dead and necrotic cells were determined according to the color of the cells and their chromatin organization as described by Coligan et al.[17]

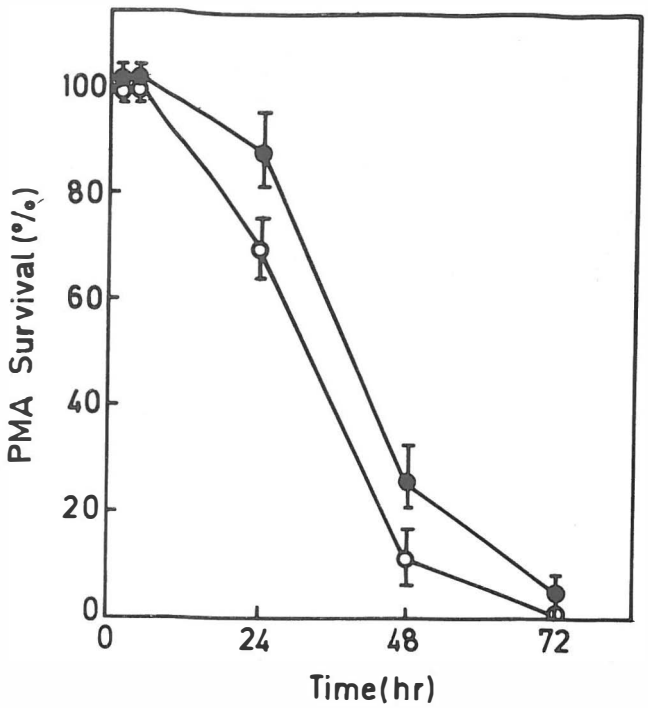

Fig. 1. Survival curve of human PMNs in cultures containing 20% FCS (●) and without FCS (○). Results are the mean of three analyses performed in duplicated, at 24 hours of incubation ($p<0.01$).

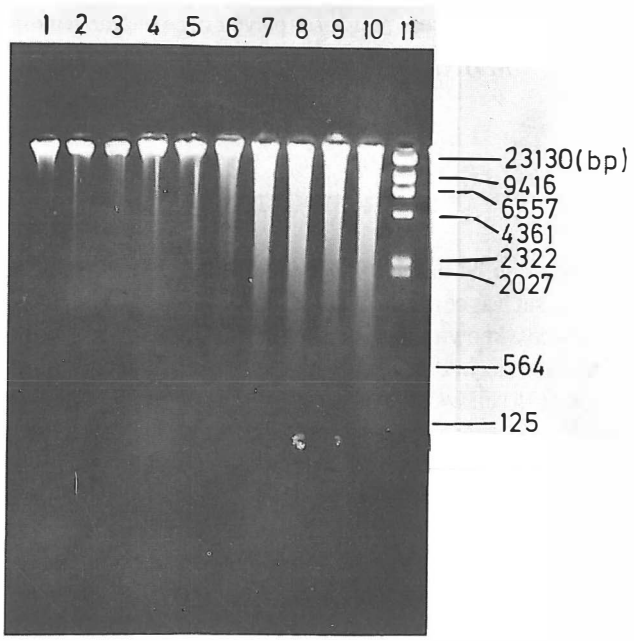

Fig. 2. Agarose gel (1.2%) electrophoresis of DNA obtained from the incubation of PMNs in the presence and absence of 20% FCS in medium. Even lanes are without serum. Incubation times are 0, 3, 6, 18 and 24 hours. Lane 11 is ECOR1 digested standard DNA.

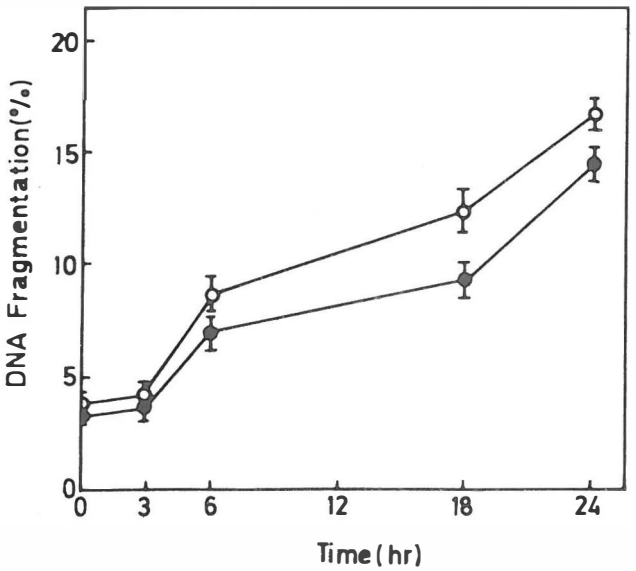

Fig. 3. Percent of DNA fragmentation; FCS deprived (○); +20% FCS (●). Results are the mean of three analyses performed in duplicate, at 24 hours of incubation ($p<0.01$).

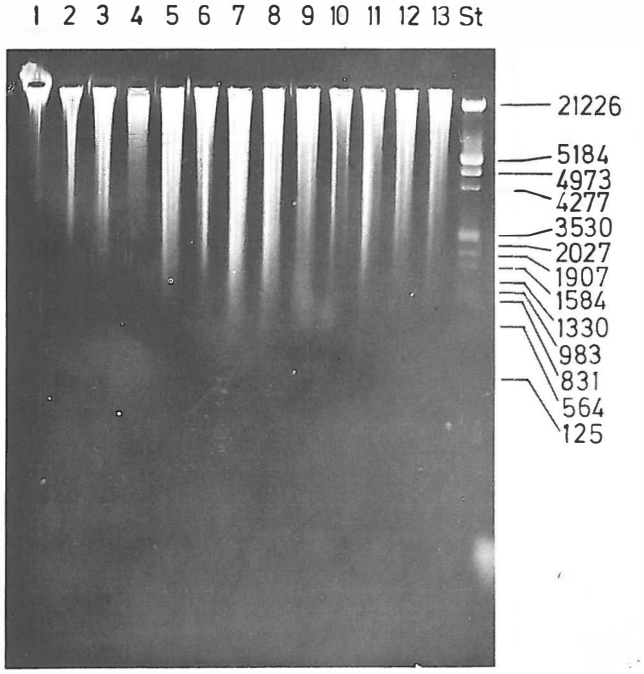

Fig. 4. Agarose gel electrophoresis of DNA isolated from PMN cells incubated with actinomycin D and cycloheximide and untreated cells (control). Lanes 1-5 are DNA from untreated cells (control), lanes 6-9 are cultures containing 20 µg/mL actinomycin D and lanes 10-13 are in the presence of cycloheximide (20 µg/mL).

DNA fragmentation analysis

After various times of incubation, treated cells and controls were lysed in 0.5 mL hypotonic buffer containing 10 mM Tris-HCl, 1mM EDTA, 0.2% Triton X-100, pH= 7.4 and released DNA fragments were determined by diphenylamine as described by Coligan et al.[17]

DNA extraction

Pellets from actinomycin D and cycloheximide treated cells and the controls were disrupted in a buffer containing 200 mM Tris-HCl, 100 mM EDTA, 1% SDS and 50 µg/mL proteinase K, pH= 8 and incubated for 4 hr at 37°C.[18] After incubation, DNA was extracted twice, each with one volume of phenol, chloroform, and isoamyl alcohol (25: 24: 1 v/v/v) and then precipitated with two volumes of 96% ethanol. Precipitated DNA was washed once with 70% ethanol and then air-dried. DNA samples were dissolved in TE buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH= 8. The DNA concentration in each sample was determined by measuring the absorbance at 260 nm using the standard curve.

Agarose gel electrophoresis

To each sample, 2 µL of loading buffer containing 30% Ficoll-400, 0.25% bromophenol blue, and 0.25% xylene cyanol was added and warmed for 10 min at 65°C and then loaded onto 1.2% agarose gel. The gel was run at 1 volt/cm for 17 hours at room temperature, stained with 0.5 µg/mL ethidium bromide for 30 min and visualized under a portable UV-lamp.

RESULTS

Examination of the isolated PMNs under the microscope showed that the cells were more than 97% pure and 99% viable. Fig. 1 illustrates the survival curve of the PMN cells cultured in the presence or absence of FCS at various times of incubation. As shown, in serum containing cultures, viability is 95% until 12 hours of incubation and afterwards starts to decrease. At 24 hours, 90% of the cells were viable but at the end of 48 h only 27% viability was observed. On the other hand, in serum withdrawal cultures, although the curve represented a similar pattern, the extent of decrease in viability was different as after 24 hours of incubation only 70% of the cells were viable.

Apoptotic, necrotic and dead indices were determined by using fluorescent dyes of acridine orange and ethidium bromide. The result is presented in Table I. As seen, in the medium without FCS the indices were higher than when they were cultured in the presence of 20% FCS. Also, there was a significant difference between the two dead indices after 24 hours of incubation ($p<0.01$).

To find out when apoptosis begins in serum withdrawal cultures, DNA was isolated from the cultures at different time intervals starting from zero to 24 hours.

The results are shown in Fig. 2. As seen, in both conditions and at zero time, DNA represents a thick band

Table I. Effect of 20% FCS on the viability indices of PMNs.

Time (hr)	+ FCS 20%			-FCS		
	AI	NI	DI	AI	NI	DI
0	1.67±0.7	0	0	1.97±0.34	0	0
3	5.03±0.33	0.33±0.25	0.46±0.41	6.64±1.13	0.6±0.14	0.86±0.23
6	13±0.29	0.77±0.26	1.6±0.28	18.6±0.81	1.4±0.37	2.13±0.4
18	39.8±2.8	1.16±0.40*	2.6±0.75*	46.18±3.4	3.56±0.33	7.97±1.11
24	57.9±1.33*	2.67±0.61*	7.73±1.39*	65.33±1.99	7.03±0.41	13.5±0.14

AI: apoptotic index; NI: necrotic index; DI: dead index.
Data are the mean of 5 experiments ± standard deviation.
* $p<0.05$

Table II. Percent of DNA fragmentation in PMNs obtained from the DPA reaction procedure.

Time (hour)	Cycloheximide (20 µg/mL)	Control	Actinomycin D (20 µg/mL)
0	4.5±0.30	4.0±0.44	4.3±0.15
3	4.98±0.89	4.6±0.6	6.12±0.83
6	11.1±0.70**	8.42±1.2	12.46±0.41*
18	14.8±0.99*	10.97±0.93	16.79±1.45*
24	20.34±1.01*	15.5±0.68	23.7±0.87*

Data are the mean of 3 experiments ± standard deviation performed in duplicate. *$p<0.01$, **$p<0.05$

with low mobility. However, DNA samples after 6 hours of incubation showed excessive smear (lane 6) in the FCS-free medium compared to DNA samples obtained from FCS containing medium (lane 5). After 6 hours of incubation DNA obtained from both media presented an increased level of smears. Smears indicate DNA fragmentation, occuring during apoptosis. Therefore, it was necessary to determine the percent of DNA fragmentation. For this purpose, reactions were performed at various incubation times. The results given in Fig. 3 confirmed the agarose gel electrophoresis pattern, indicating that the percentage of DNA fragmentation in FCS-treated cells was lower than that of untreated cells at all incubation times.

The effect of non-toxic concentrations of actinomycin-D and cycloheximide on the apoptosis of PMNs was investigated under serum withdrawal conditions. Fig. 4 shows the agarose gel electrophoresis pattern of DNA isolated from actinomycin D or cycloheximide-treated cells and the controls. Actinomycin D-treated samples produced a typical ladder pattern of internucleosomal cleavage in culture after 6 hours of incubation (lanes 6-9), which was an indicator of accelerated apoptosis. However, the onset of apoptosis was low in the control and no ladder was obtained. The effect of cycloheximide on PMN cells is also shown in Fig. 4 (lanes 10-13). It is clear that cycloheximide also increased the rate of apoptosis, but the extent of this increase was less than that obtained for actinomycin D.

Table II gives the percent of DNA fragmentation obtained from the diphenylamine reaction procedure. As is seen, although the control also shows fragmentation, DNA from the cells treated with actinomycin D (20 µg/mL) or cycloheximide (20 µg/mL) showed a high level of DNA fragmentation.

DISCUSSION

Apoptotic cell death is a widespread biological phenomenon implicated in homeostatic control of the number of cells in the mammalian body. However, the molecular mechanisms underlying naturally occurring cell death are not well understood.

Our results presented here demonstrate that when fetal calf serum is removed from human neutrophilic granulocyte cultures, PMN survival decreases with increasing periods of incubation, thus apoptosis is accelerated in serum-withdrawal culture after 6 hours of incubation. In contrast, in the presence of serum, apoptosis begins after 6 hours and needs further incubation in order to progress.

Results obtained from the measurements made by fluorescent dyes and the diphenylamine reaction confirm the above data. Treating PMNs with two inhibitors of protein synthesis–actinomycin D and cycloheximide–accelerates apoptosis, although actinomycin D acts more rapidly compared to cycloheximide. Both induce pronounced changes in the nuclear chromatin leading to the breakdown of chromatin into internucleosomal fragments.

The cleavage of chromosomal DNA into oligonucleosomal-sized fragments has been repeated in PMN cultures with 10% serum after 12 hours of incubation,[19] while our results show the typical ladder at 6 hours of incubation with both factors in serum withdrawal culture.

These results, together with those reported by others,[20]

suggest that spontaneous apoptosis of neutrophils may usually be inhibited by synthesized protein. Nevertheless, it has been shown that protein synthesis is a prerequisite for indicating apoptosis in other systems.[21] On the other hand, these agents may induce neutrophil apoptosis not by inhibiting the synthesis of proteins which prevent apoptosis, but by direct provocation similar to other apoptosis agents.

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