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The ordered disintegration of nuclear DNA as a specific genome reaction accompanying apoptosis, stress response and differentiation

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The treatment of agarose embedded nuclear or cellular preparations with protein denaturing agents resulted in ordered cleavage of intact nuclear DNA into high molecular weight fragments with the pattern of fragmentation being unityped for various eukaryotic representatives. We showed that the set of DNA fragments represents the pre-existing DNA structural domains attributed to the higher levels of chromatin folding, and presented evidence allowing to interpret the nuclear DNA domain organization as a constituent component of topoisomerase II/DNA complex with its ability to mediate the cleavage/religation reactions. We demonstrated that changes in the integrity of nuclear DNA, recognizable as an altered pattern of SDS-dependent cleavage of nuclear DNA into high molecular weight DNA fragments, took place at the early stage of apoptosis, upon number of stress challenges and in cells showing various proliferative status. The changes in the integrity of nuclear DNA affected by various influences were shown to be prompt and seem to be of transient nature. The results obtained allow to conclude that changes in the integrity of nuclear DNA revealed as an altered pattern of SDS-dependent high molecular weight DNA cleavage may present the specific genome reaction accompanying the physiological changes in the cells during apoptosis, stress response and differentiation.

Introduction. Genomic DNA within eukaryotic cell nucleus appears to be organized into loop domains sized about 40—100 kb, which are fixed on the protein backbone structure referred to as nuclear matrix or chromosome scaffold [1—11]. Topoisomerase II have been shown to be a major component of the nuclear matrix and chromosome scaffold fraction and play important role in chromosome structure and condensation and in genome expression [12—14]. Several lines of evidence suggest that type II enzyme appears to be concentrated in a number of discrete anchoring complexes, which probably form the basis of the chromatin loop domains [15, 16]. The organization of nuclear DNA into loop domains provides reasons to believe that each structural domain may correspond to individual functional genome unit, which is compartmentalized

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relative to regulatory elements by DNA sequences that are bound to the nuclear matrix [17—22] (for review see [19]). Several investigators reported that changes in DNAse I sensitivity that can extend over several dozens of kilobases accompany changes in expression of specific genes [23, 24] (for review see [3]), with the boundaries of transcriptionally active nuclease-sensitive domains being commonly coincided with matrix attachment regions [25—28]. In a number of studies using a variety of fractionation techniques both replicating DNA and transcriptionally active sequences were shown to exhibit an altered interaction with nuclear matrix [11, 29—35] (for review see [19, 33—35]). These data indicate that some functionally significant structural rearrangements of chromatin may occur during DNA replication and transcription.

Recently it was shown that the formation of discrete set of high molecular weight (HMW) DNA fragments proceeds at the early stages of apoptosis [36, 37], which may be interpreted as a key event involved in the programmed cell death [38, 39]. This finding suggests that the ordered disintegration of nuclear DNA into HMW-DNA fragments may represent an early event in specific cell programme resulting in cell self-demise.

In the present investigation we report data to show that the changes in the integrity of nuclear DNA accompany various cell programmes including apoptosis, stress response, proliferation and may be interpreted as a specific genome reaction being of physiological value.

Material and Methods. *Cell lines and culture conditions.* The human lymphoblastoma cultured cells (line CEM) and primary cell culture of murine thymocytes obtained from the thymus of 4—5 week old mouse (line BALB/c) were used for investigation of apoptosis and stress response. Human cultured cells were routinely incubated in RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS) in atmosphere of 95 % air, 5 % CO_2 to give a final suspension of $5 \cdot 10^6$ cells/ml. Thymocyte primary culture was prepared from intact thymocytes to final suspension of $2 \cdot 10^7$ cells in RPMI 1640/10 % FCS and incubated under conditions indicated above for at least 6 h either with or without apoptotic inducers. Apoptosis was induced by incubation of cells either with 1 μM dexamethasone or 7.5 μM teniposide (VM-26). The cell survival fraction was estimated by Trypan blue exclusion.

Preparation of cellular samples to FIGE fractionation. 200 μl of cell suspension were placed into the well of cell culture plate followed by addition of equal volume of 1 % low-melting point agarose prepared on TEN-buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl). After gelation the equal volume of lysing buffer (TEN + 1 % SDS) was layered followed by incubation for 1 h at 37 °C. Agarose plugs containing the lysed cells were used for analysis by agarose gel electrophoresis.

Gel electrophoresis. Lysed cell preparations were fractionated either by conventional or field inversion gel electrophoresis (FIGE) to detect the pattern of nuclear DNA cleavage. Conventional gel electrophoresis was carried out in 1.4 % agarose at 50 V for 4—5 h using 0,5xTBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.5). FIGE was performed in 1 % agarose at 85 V for 18 h in 0,5xTBE buffer under constant pulses of electric field (24 s «forward» and 8 s «backward») allowing to monotonous resolution of DNA molecules sized up to 500 kb [50]. In some cases FIGE was carried out for 5—6 h allowing to resolve both low- and high molecular weight DNA. After electrophoresis the gel was stained with 1 μg/ml ethidium bromide for 10 min, viewed using UV transilluminator and photographed using Mikrat 300 film.

Results and Discussion. We showed in our previous works that the fractionation of agarose embedded nuclei samples, treated with SDS, by field inversion gel electrophoresis (FIGE) results in appearence of two main types of discrete DNA fragments sized about 50—100 kb and 250—300 kb, with the

pattern being comparable for various eukaryotic representatives [40]. The treatment of nuclei preparations with protein denaturing agents represents the decisive prerequisite for ordered HMW-DNA cleavage. No DNA fragment is released into gel provided these agents lacking even if nuclei were destroyed with high concentration of EDTA [40].

Re-fractionation of nuclear preparations by FIGE with various pulsed field switching intervals or by orthogonal field alteration gel electrophoresis (OFAGE) showed that the group of DNA fragments of about 250—300 kb contributes to the limited mobility zone under FIGE employed while the another group of DNA represents the set of DNA fragments with the real size of about 50—100 kb (results not shown).

As evidenced from the data presented in Fig. 1, the pattern of SDS-dependent HMW-DNA cleavage is similar to that found in fractionated cell-, nuclei- and «nucleoid» preparations. The only difference is that in the nucleoids DNA fragments of about 50—100 kb represent the major DNA cleaved product. The maintaining of an ordered nuclear DNA fragmentation in nucleoids (histone-depleted nuclei) suggests that the HMW-DNA fragments revealed by FIGE may be relevant to the higher-level DNA structural organization. Based on the data of structural organization of histone-depleted nuclei, it is conceivable to ascribe the observed 50—100 kb DNA fragments to DNA loop domains the average length of which varies between 40—100 kb [10, 41].

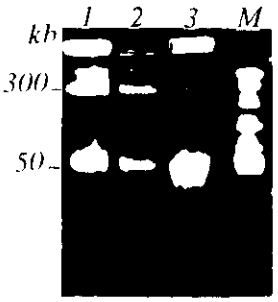

Fig. 1. The pattern of lymphocytes nuclear DNA fragmentation in cells (*1*), nuclei (*2*) and nucleoids (*3*) preparations. Human lymphocytes were collected by centrifugation, resuspended in TE-buffer containing 0.15 M NaCl and embedded into low-melting agarose. After gelation samples were treated with 1 % SDS followed by FIGE fractionation. For preparation of nuclei, cells were resuspended in buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 % triton X-100, 0.32 M sucrose. After centrifugation nuclear pellet was resuspended in the same buffer without triton followed by addition of equal volume of 1 % agarose. Nucleoids (histone-depleted nuclei) were prepared by extensive nuclei treatment with 2 M NaCl followed by embedding into agarose. After gelation nuclei and nucleoid preparations were treated with 1 % SDS and fractionated by FIGE. *M* — molecular weight standards, lambda DNA oligomers

Data presented in Fig. 2 show that nuclear DNA structural domains seem to be involved in functioning topoisomerase II/DNA complex with the main ability to carry out cleavage/rejoining reactions. Thus, the specific modulator of topoisomerase II activity, teniposide (VM-26), enhances the ordered HMW-DNA cleavage which results in the increased amount of DNA released into gel and in breaking down of the large DNA fragments into 50—100 kb ones. (Fig. 2, *B*, line *1*, *2*). On the other hand, the conditions provoked by the

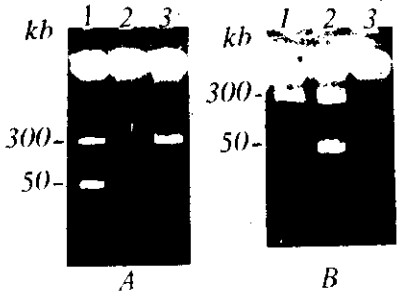

Fig. 2. Modulation of topoisomerase II-mediated cleavage/religation reactions in cultured human cells: *A* — cultured lymphoblastoma cells (line CEM) were incubated at 37 °C (*1*), subjected at 4 °C for 30 min (*2*) followed by incubation at 37 °C for 30 min (*3*); *B* — the same cells were incubated at 37 °C without (*1*) or with 7.5 μM teniposide (*2*) for 20 min followed teniposide treatment the cells were incubated at 55 °C for 10 min (*3*) (*A*, *B* — after incubation cells were embedded into agarose, treated with SDS and fractionated by FIGE)

topoisomerase II-mediated reverse reaction lead to the rejoining of cleaved DNA domains (Fig. 2).

The ordered disintegration of nuclear DNA into HMW-DNA domains seems to be of physiological value since the formation of HMW DNA fragments may be involved in the early events accompanying programmed cell death. This is evidenced from the data presented in Fig. 3, which show that the

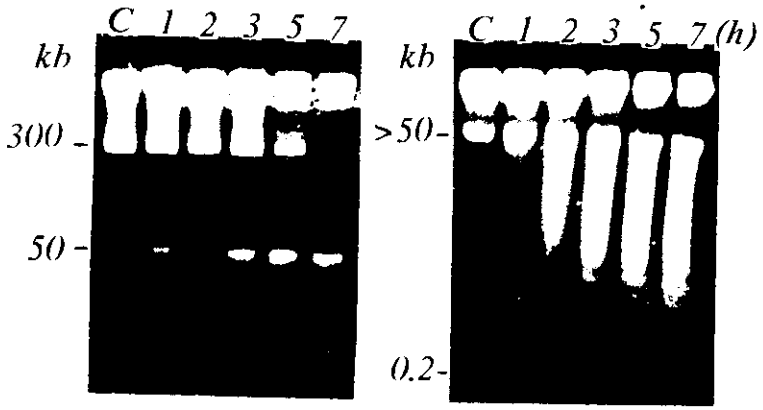

Fig. 3. Gel electrophoretic analysis of nuclear DNA cleavage during dexamethasone induced apoptosis in murine thymocytes. Cells were treated with 1 μM dexamethasone and allowed to incubate for the time indicated at the top of the Figure. After incubation cells were embedded into low-melting point agarose, treated with SDS and fractionated either by FIGE (top panels) or conventional gel electrophoresis (bottom panels). *C* — control cells (not treated with dexamethasone)

dexamethasone induced apoptosis in primary culture of thymocytes is associated with enhanced nuclear DNA cleavage into fragments of about 50—100 kb, which is detectable at the early stage of apoptosis and precedes typical for apoptosis internucleosomal DNA fragmentation. It seems likely, however, that the ordered HMW-DNA cleavage is not restricted to apoptotic cell death. The data presented in Fig. 4 show that *in vitro* incubation of primary culture of thymocytes without apoptotic inducers is accompanied with the rapid increase of nuclear HMW-DNA cleavage to be kept «frozen» without typical for apoptosis oligonucleosomal «ladder» development. These data suggest that ordered

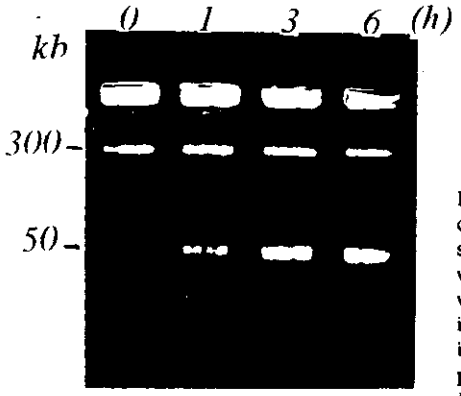

Fig. 4. Gel electrophoretic analysis of nuclear DNA cleavage in primary culture of thymocytes non-stimulated to apoptosis. Intact murine thymocytes were resuspended in RPMI medium supplemented with 10 % FCS and allowed to incubate for the time indicated at the top of the Figure (h). After incubation cells were embedded into low-melting point agarose, treated with SDS and fractionated by FIGE

disintegration of nuclear DNA revealed as an enhanced HMW-DNA cleavage contribute not only to the apoptosis but may be implicated in cell response to stress challenges induced by thymocyte introduction into *in vitro* culture. As a confirmation to this may be data presented in Fig. 5, indicating that both

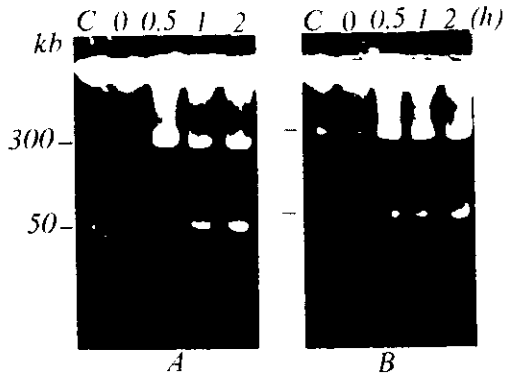

Fig. 5. The pattern of ordered DNA cleavage in cultured CEM cells in response to cold shock. Cells were incubated for 10 min at 4 °C (*A*) or 1 h at 15 °C (*B*) followed by incubation at 37 °C for the time indicated at the top of figure (h). After incubation cells were embedded into low-melting point agarose, treated with SDS and fractionated by FIGE. C — control cells not subjected to shock

short-time acute and prolong gentle cold shock appear to induce in cultured human lymphoblastoma cells (line CEM) rapid changes in the pattern of HMW-DNA cleavage which is invariably persistent for at least 2 h recovering after cold shock.

As evidenced from the data presented in Fig. 6 the ordered disintegration of nuclear DNA into HMW-DNA fragments may be of transient nature. Thus, cultured CEM cell incubation in serum-free medium is accompanied by the increased formation of HMW-DNA fragments to be rapidly declined following serum addition and practically disappeared after 24 h incubation with fresh medium with serum (Fig. 6). Similarly, the dilution of monolayer HeLa cells with fresh medium is also associated with rapid changes in the pattern of SDS-dependent HMW-DNA cleavage (Fig. 7). The generation of HM W-DNA fragments is progressively decreased during cell incubation in fresh medium and completely disappeared after 24 h of incubation. However, after 2/3 of new monolayer has been established, the formation of HMW-DNA is resumed (Fig. 7).

These data indicate that changes in the pattern of SDS-dependent HMW-DNA cleavage, seem to be of transient nature, and may be implicated not only in cell response to stress challenges but also to accompany the changes in the proliferation status of the cell. It allows to interpret the changes in the integrity of nuclear DNA structural domains as a specific genome reaction being of physiological value.

Our data show that the treatment of agarose-embedded nuclear or cellular preparations with SDS results in ordered fragmentation of nuclear DNA into HMW-DNA fragments with the pattern of fragmentation being comparable for various eukaryotic representatives. The maintaining of an ordered nuclear DNA fragmentation in histone-depleted nuclei (Fig. 1) suggests that the formation of HMW-DNA fragments appears to be independent on the presence of histones and may be due to the periodicity of DNA folding in the cell nucleus. This provides reasons to believe that HMW-DNA fragments revealed by FIGE fractionation of SDS treated nuclear preparations represent pre-existing structural domains to reflect the higher levels of chromatin organization.

Based on the data of structural organization of histone-depleted nuclei, it

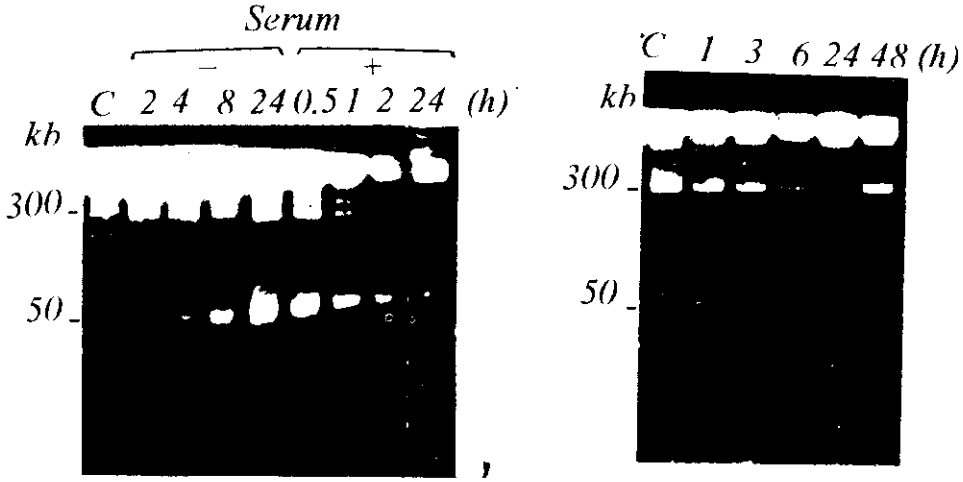

Fig. 6. The transient nature of ordered DNA cleavage in cultured CEM cells during serum starvation. Cells were incubated in serum-free medium (–) for the time indicated at the top of figure (h). Following starvation serum was added and cells were incubated in serum-containing medium (+) for the time indicated at the top of figure (h). After incubation cells were embedded into agarose, treated with SDS and fractionated by FIGE. *C* — control cells before serum starvation

Fig. 7. The transient nature of ordered DNA cleavage in cultured HeLa cells. Monolayer cells were washed with cultural medium to remove dead cells and treated with tripsine to obtain cellular suspension. Portion of cellular suspension was embedded into agarose to analyse nuclear DNA cleavage (control cells — *C*), while the equal another portions were supplemented with fresh medium and allowed to incubate for the time indicated at the top of figure (h). After incubation cells were washed with cultural medium to remove dead cells, tripsinized, collected by centrifugation and embedded into agarose followed by SDS treatment and FIGE fractionation

is conceivable to ascribe the 50—100 kb DNA fragments to DNA loop domains the average length of which varies between 40—100 kb [10, 41]. Similar results were obtained by other authors as well [42—44]. As far as 250—300 kb DNA fragments are concerned these contribute to the limited mobility zone under FIGE employed and may be interpreted as a heterogeneous population of DNA fragments consisting of noncleaved loop domains. This is supported by the fact that these fragments may be converted into 50—100 kb ones by a number of influences including topoisomerase II specific poisons [40]. The sensitiveness of SDS-dependent HMW-DNA cleavage to topoisomerase II-specific poison teniposide and rejoining of cleaved HMW-DNA domain under conditions specifically stimulating the topoisomerase II-mediated religation reaction (Fig. 2) add credence to the idea that the nuclear DNA structural domains are involved in functioning topoisomerase II/DNA complex the main property of which is its ability to mediate the cleavage/rejoining reactions.

Our data evidence that the pattern of SDS-dependent HMW-DNA cleavage may follow the physiological changes in the cells thus suggesting that changes in the integrity of nuclear DNA may be of physiological value. Thus, the enhanced HMW-DNA cleavage was shown to be the early event involved in programmed cell death. Comparable data were obtained recently by other investigators [36—39] to demonstrate that the formation of HMW-DNA fragments may be considered as a biochemical hallmark of apoptosis.

Our results indicate that changes in the integrity of nuclear DNA revealed as an altered pattern of SDS-dependent HMW-DNA cleavage occur not only during apoptosis but upon cell challenging with stress stimuli (Fig. 3—6). Since any stress influence may interfere with cellular viability one should believe that the increased HMW-DNA fragmentation may reflect the fact that cells become predisposed to apoptosis. The same reason may be for the monolayer HeLa cells, which show the increased formation of HMW-DNA fragments (Fig. 7).

However, our data show that the ordered disintegration of nuclear DNA in apoptotic thymocytes seems to be uncoupled with apoptosis-specific internucleosomal DNA cleavage and takes place in those thymocytes not stimulated to apoptosis (Fig. 4). In addition, the quite different pattern of HMW-DNA cleavage may be observed even in the same plant tissues showing various differentiation level (like shoots and roots of pea seedlings). As evidenced from the results in all cases under study there is a tendency to enhanced HMW-DNA fragmentation in quiescent or terminally differentiated tissues, which is revealed as a changed proportion between the two types of DNA fragments towards 50—100 kb fragments increase [51].

These results suggest that HMW-DNA cleavage seems to be unrelated with apoptosis-specific nuclear DNA degradation, but more likely represent the cellular response being of physiological value. This is further supported by the observation that formation of HMW-DNA fragments seems may be of transient nature (Fig. 6, 7). Taken together our results suggest that there may be a specific cellular response resulting in structural rearrangements of nuclear DNA domains, which accompany the physiological changes in the cells during various cellular programmes including apoptosis, stress response and differentiation. Our interpretation of ordered HMW-DNA cleavage is based on previously obtained data showing that nuclear DNA structural domains are involved in functioning topoisomerase II/DNA complex [45]. We demonstrated that the properties of this complex are comparable with those described for *in vitro* established complex purified enzyme/plasmide DNA [46, 47]. The main property of this complex was shown to be its ability to mediate the cleavage/rejoining reactions of DNA structural domains ([45]; Fig. 2, present communication). Based on the studies with purified topo II enzymes and DNA the two-stage model for topoisomerase II-mediated cleavage/religation reactions has been proposed [46, 47]. According to this model an enzyme/DNA cleavable complex is the key covalent intermediate in the topoisomerase II mediated DNA turnover, being in rapid equilibrium with noncleavable complex [46—48]. The exposure of the cleavable complex but not noncleavable one to protein denaturants (such as SDS or alkali) results in cleaved DNA product involving the covalent linking of topoisomerase II subunits to the 5'-ends of broken DNA [46, 47]. Proceeding from the results that structural domains of nuclear DNA contribute to the functioning topoisomerase II/DNA complex [45] it seems appropriate to interpret the ordered disintegration of nuclear DNA into HMW-DNA fragments as DNA structural domain turnover from «noncleavable» to «cleavable» state, mediated by topo II enzymes. Thus, our results allow to conclude that changes in the integrity of nuclear DNA revealed as an altered pattern of SDS-dependent HMW DNA-cleavage may present the specific genome reaction accompanying the physiological changes in the cells. This reaction may be interpreted as the turnover of structural domains between «cleavable» and «noncleavable» state whose physiological value remains to be elucidated.

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Упорядкована дезинтеграція ядерної ДНК як специфічна геномна реакція, що супроводжує апоптоз, відповідь на стрес і диференціювання

Резюме

У даній роботі показано, що при дії на «заплавлені» в агарозу препарати клітин та ядер білкових денатурантів відбувається упорядковане крупноблочне розщеплення інтактної ядерної ДНК. Фрагменти, що утворюються при цьому, являють собою передіснуючі структурні домени

ядерної ДНК, які відповідають вищим рівням упакування хроматину. Їх можна розглядати як конститутивний компонент ядерного комплексу ДНК/топоізомераза II, спроможного здійснювати реакцію розщеплення — воз'єднання ДНК. Встановлено, що зміна нативності, або цілісності, ядерної ДНК, яка виявляється у зміні характеру DS-Na-залежного крупноблочного розщеплення ядерної ДНК, відбувається на ранніх етапах апоптозу під дією різноманітних стресових факторів, а також у клітинах з різним рівнем проліферативної активності. Отримані результати дозволяють припустити, що спостережені зміни нативності ядерної ДНК можуть бути специфічною геномною реакцією, яка супроводжує фізіологічні процеси в клітині при апоптозі, відповіді на стрес або диференціюванні

В. Т. Соловьян, И. О. Андреев, Т. Ю. Колотова, П. В. Погребной, Д. В. Тарнавский

Упорядоченная дезинтеграция ядерной ДНК как специфическая геномная реакция, сопровождающая апоптоз, ответ на стресс и дифференцировку

Резюме

В данной работе показано, что воздействие на препараты «заплавленных» в агарозу клеток или ядер белковых денатурантов приводит к упорядоченному крупноблочному расщеплению интактной ядерной ДНК. Фрагменты, образующиеся при этом, являют собой предсуществующие структурные домены ядерной ДНК, соответствующие высшим уровням упаковки хроматина. Их можно рассматривать как конститутивный компонент ядерного комплекса ДНК/топоизомераза II, способного осуществлять реакцию расщепления/воссоединения ДНК. Установлено, что изменение нативности, или целостности, ядерной ДНК, проявляющееся в изменении характера DS-Na-зависимого крупноблочного расщепления ядерной ДНК, происходит на ранних этапах апоптоза под влиянием всевозможных стрессовых факторов, а также в клетках с различным уровнем пролиферативной активности. Эти изменения происходят быстро и могут иметь обратимый характер. Полученные результаты позволяют предположить, что наблюдаемые изменения нативности ядерной ДНК могут представлять собой специфическую геномную реакцию, сопровождающую физиологические процессы в клетке при апоптозе, ответе на стресс или дифференцировке.

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