Possible involvement of DNA strand breaks in regulation of cell differentiation

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The present review summarizes data on the accumulation of DNA strand breaks in differentiating cells. Large 50 Kbp free DNA fragments were observed by several research teams in non-apoptotic insect, mammal and plant cells. A more intensive DNA breakage was observed during maturation of spermatides, embryo development, and differentiation of myotubes, epidermal cells, lymphocytes and neutrophils. In general, accumulation of DNA strand breaks in differentiating cells cannot be attributed to decrease of the DNA repair efficiency. Poly(ADP)ribose synthesis often follows the DNA breakage in differentiating cells. We hypothesize that DNA fragmentation is an epigenetic tool for regulation of the differentiation process. Scarce data on localization of the differentiation-associated DNA strand breaks indicate their preferred accumulation in specific DNA sequences including the nuclear matrix attachment sites and repeats. Recent data on non-apoptotic functions of caspases provide more evidence for possible existence of a DNA breakage mechanism in differentiating cells resembling the initial stage of apoptosis. Excision of methylated cytosine and recombination are other possible explanations of the phenomenon. Elucidation of mechanisms of differentiation-induced DNA strand breaks appears to possess considerable research potential.

Key words: differentiation, DNA breaks, poly(ADP)ribosylation, deamination, demethylation.

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Evidence for spontaneous fragmentation of DNA in eukaryotic cells

The arrival of the post-genomic era is marked by increasing interest in epigenetic mechanisms of genome regulation. In the present review we would like to remind the scientific community about the existence of a probable regulatory function for DNA strand breaks. This concept was developed in the eighties, but later the importance of these findings was shadowed by the boom in apoptosis research. Normal functional activity of the cell is inevitably followed by formation of DNA strand breaks or by modifications of DNA structure that can be registered as DNA lesions. Meiotic recombination and V(D)J-recombination in differentiation of lymphocytes are impossible without formation of DNA strand breaks (de la Roche Saint-Andre, 2005). Moreover, DNA is permanently damaged by normal cell metabolites (Marnett, Plastaras, 2001). It is considered that up to 50 single-strand breaks are formed in each mammalian cell per minute, this gives up to 3 000 breaks per cell cycle, resulting in formation of 50 double-strand breaks per cycle (Vilenchik, Knudson, 2000; 2003). DNA replication and action of topoisomerases is also followed by interruptions of DNA integrity (Eastman, Barry, 1992). Some authors believe that eukaryotic DNA is interrupted by protein (Szabo, 1995) or lipid (Struchkov *et al.,* 2002) linkers. Interruptions in DNA structure leads to formation of free 50-100 Kbp fragments (Solov'yan *et al.,* 1997). The concept of *forum DNA* revealed as a fast-migrating fraction during pulse-field electrophoresis assumes that the fraction is formed due to inevitable incisions performed by endonucleases in the course of DNA deproteinisation (Tchurikov, Ponomarenko, 1992; Tchurikov *et al.,* 1998; Tchurikov *et al.,* 2004). DNA fragments of 50 Kbp were revealed in proliferating cultured mammalian cells of different origin with no signs of cell death (Szabo, 1995). Moreover, the same team has proved the existence

of a single-strand break on every 50 Kbp (Varga *et al.,* 1999), these breaks can be revealed by TUNEL-assay if the preparation is treated by protease, indicating existence of the protein linkers (Gal *et al.,* 2000). Spontaneous double strand DNA breaks were identified in U937 and Molt-4 cell cultures using a TUNEL assay modified for electron microscopy (E. Falcieri, personal communication). One of the authors has observed individual peculiarities in number of single-strand and double-strand DNA breaks as well as large-scale fragmentation of DNA in Xenopus erythrocytes; blood was taken from alive animals, and the pattern of DNA fragmentation was reproduced in sequential analyses (Figure 1). On Figure 1a, lane 2 fraction of small single-stranded DNA fragments is clearly visible, it was never observed in another animal (lane 1), it means that erythrocyte DNA of animal 2 contains more single-strand DNA breaks compared to DNA of animal 1. Low-voltage electrophoresis in neutral conditions indicates that DNA of two animals (Figure 1b, lanes 2 and 3) contains a fast-migrating fraction absent in the third animal (lane 4). In some toads, pulse-field electrophoresis revealed a fast-migrating fraction (300 Kbp.–2000 Kbp; Figure 1d, lane 2), however this fraction was never observed in other individuals (Figure 1d, lane; Sjakste, 1997). Thus DNA in eukaryotic cells always contains a distinct number of DNA strand breaks; later we will try to analyze if these breaks can have some functional significance.

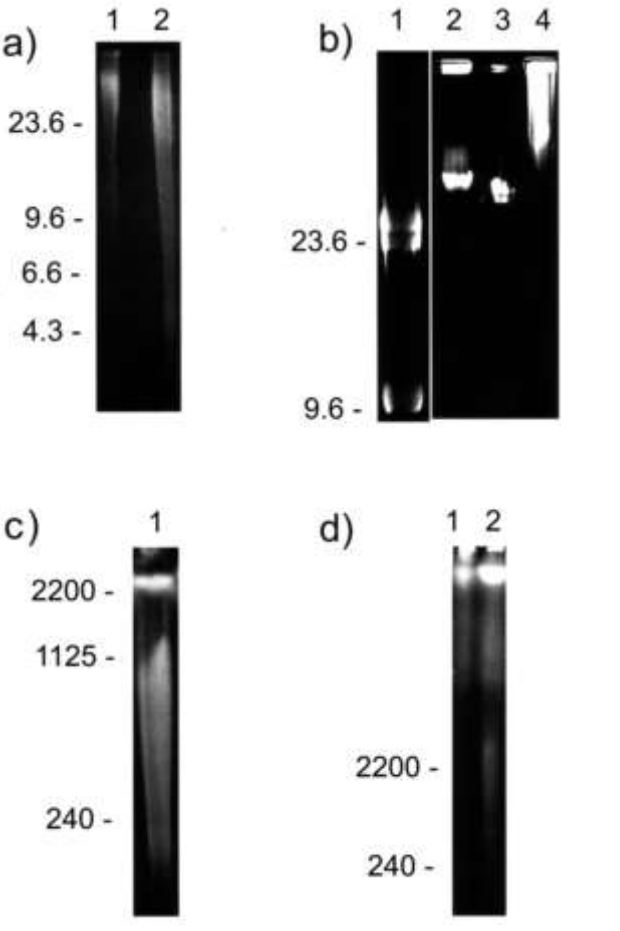

Figure 1. Evidence for DNA fragmentation in Xenopus erythrocytes. a – cells were embedded in agarose blocks and subjected to alkaline conventional electrophoresis (0.8% agarose; 30 mM NaOH, 1 mM EDTA, 30 V, overnight). 1, 2 – DNA of two animals; b - cells were embedded in agarose blocks and subjected to low-voltage electrophoresis in neutral conditions (0.75% agarose in TBE; 0.6 V/cm, 72 h at room temperature; 2, 3, 4 – DNA of three animals). c, d – pulse-field electrophoresis performed on a contour-clamped homogenous electric field (CHEF) device system in different conditions. c - 220 V, 0.8% agarose, 120° reorientation angle, 60 s switch time for 15 h followed by 90 s switch time for 8 h. d – 50 h with 600s switch time followed by 70 h with 600-2700 s switch time. 1,2 - samples from two animals. Positions of the size markers (Kbp) are given on the left. Details in (Sjakste, 1997).

DNA strand breaks in differentiation

Germinal cells

Human sperm cells contain about 12.7 times more breaks and other lesions compared to somatic cells, namely lymphocytes (Muriel *et al.,* 2004). In spermatocytes (diploid precursors of spermatids) the maximal number of DNA strand breaks is observed in early stages of development (Joshi *et al.,* 1990). Expression of phosphorylated H2AX histone, a DNA double-strand break marker, in intact mouse spermatocytes also indicates spontaneous DNA breakage in these cells (Forand *et al.,* 2004). In rat spermatids the number of nicks in DNA increases in early stages of development, reaching a maximum in the middle stages and decreasing later (Iseki, 1986). The recent study performed on murine and human spermatids indicates that DNA breakage follows replacement of histones by protamines - the breaks are necessary to remove superhelicity of the DNA molecule (Marcon, Boissonneault, 2004). These are predominantly double-strand DNA breaks generated by topoisomerase II, the process is dependent on histone H4 hyperacetylation (Laberge, Boissonneault, 2005). Andrologists manifest great interest in the studies of DNA strand breaks in the spermatozoa, as increased number of persisting DNA strand breaks is coupled to male infertility. Probably DNA strand breaks found in mature sperm are due to incomplete repair of the functional DNA strand breaks in spermatids. Persistence of theses breaks

causes the so-called abortive apoptosis, as some data indicate similar mechanisms of DNA breakage in sperm to apoptotic DNA breakage (Muratori *et al.,* 2006).

Embryo development

In sea urchin embryo significant nick number is detected on morula stage, the breaks are localized predominantly in the histone gene chromatin domains (Wortzman, Baker, 1989). In a thorough study by Zaraiskii and colleagues (1989) the spatial and temporal patterns and quantities of nicks in nuclear DNA during gastrulation and neurulation were studied using nick-translation in sections of Xenopus laevis embryos. Specific changes in the number of nicks in different mesoderm and ectoderm regions were detected during embryogenesis. A dorso-ventral gradient in labelling of nuclei was observed in the mesoderm and inner ectoderm layer of early and middle gastrula. The gradient was inverted during transition from gastrula to neurula. At the same time dorso-ventral (in mesoderm) and ventro-dorsal (in outer ectoderm layer) gradients of nuclear labelling were increased. The intensity of nuclear labelling in all parts of the embryo as a whole was remarkably higher during neurulation as compared with gastrulation. A dorso-ventral gradient of nuclear labelling was observed in mesoderm and ectoderm during neurulation.

In mouse preimplantation embryos, the chromosome reactivity in nick translation was highest at the blastocyst stage and varied according to cleavage divisions of the zygote. No gaps were observed in postimplanation embryos (Patkin *et al.,* 1995).

In murine teratocarcinoma cells, the F9 that are used as a model of early development, DNA strand breaks were observed mostly in undifferentiated cells; the breaks were repaired after induction of differentiation with retinoic acid (Kisliakova *et al.,* 2000). Induction of differentiation of embryo stem cells and culture of murine teratocarcinoma was followed by numerous DNA incisions during the 2nd to 4th mitosis after induction; the break number decreased and reached the initial level on following stages of differentiation (Vatolin *et al.,* 1997).

Nerve cells

Number of DNA strand breaks in neurons is lower compared to other cells; the number of breaks does not change during the rat embryo development and during post-natal development of the animal (Mullaart *et al.,* 1990). However, comparison of different brain structures has revealed an increased number of breaks in the cerebellum Purkinje cells compared to pyramidal cells of large hemispheres (Iseki, 1986). An analogous study by Patkin *et al.* (2001) revealed subpopulations of neurons and glial cells differing in the number of DNA strand breaks. Huge DNA fragmentation was revealed in neurons of mice with an inborn defect of double-strand break repair. It is supposed that that in normal animals these breaks are induced and immediately repaired by way of intensive recombination in neurons (Gilmore *et al.,* 2000). The idea that probable somatic rearrangements in nerve cells undergoing differentiation which contribute to the generation of neuronal heterogeneity was formulated by Chun and Schatz (1999). It was based on the discovery of the recombination activating gene-1 (RAG-1) transcript in the murine central nervous system (Chun *et al.,* 1991). This idea received indirect support from experiments on deletion of genes participating in DNA recombination. Deletion of DNA ligase IV (Barnes *et al.,* 1998; Gao *et al.,* 1998), its dimerization partner XRCC4 (Gao *et al.,* 1998), and Ku proteins (Gu *et al.,* 2000) resulted in the early neuronal death and consequent loss of embryos. Thus, induction and repair of double strand DNA breaks appear to be necessary steps in neuron differentiation. p53 deficiency and ataxia-telangiectasia-mutated (ATM) gene deficiency rescue the embryonic lethality and neuronal apoptosis, (Frank *et al.,* 2000; Sekiguchi *et al.,* 2001), these features cause the difference between recombination mechanisms in lymphocytes, that are independent of p53 and ATM and probable recombination in neurons.

Muscle cells

Differentiation of the primary chicken myoblasts is followed by induction of 100 to 300 single-strand DNA strand breaks per cell (Farzaneh *et al.,* 1985). The breaks are formed in young myotubes, later these are repaired (Dawson, Lough, 1988). Muscle cell nuclei contain the highest number of DNA strand breaks of all mammalian tissues (Iseki *et al.,* 1986). Differentiation of muscle cells is followed by increase of the Ca^{2+}/Mg^{2+} - dependent endonuclease activity (Shiokawa *et al.,* 2002). Exogenous DNA breakage delays differentiation of the C2C12 myoblast culture (Puri *et al.,* 2002); on the contrary, in Drosophila myoblasts DNA break-

age triggers the differentiation process (Hossain *et al.,* 2005).

Fibroblasts

Differentiation of transformed Djungarian hamster fibroblasts in confluent culture is followed by accumulation of single- and double strand breaks that are localized presumably in a fraction of transcriptionally active chromatin (Arshavskaya *et al.,* 1989; Sjakste, Budylin, 1990).

Erythroid cells

The first two reports about the involvement of the DNA strand breaks in the erythrodifferentiation process appeared in simultaneously published articles about the accumulation of DNA strand breaks in the cells of Friend erythroleukemia induced to differentiate with dimethylsulfoxide (DMSO) (Terada *et al.,* 1978; Scher, Friend , 1978). It was also reported that exogenous breaks induced by UV-light or bleomycin also induce transition of cells to first stages of erythrodifferentiation. Other authors disagreed with these conclusions and claimed it was erroneous, the data were interpreted as follows: DMSO triggered a temporal arrest of the cells in the G1-phase of the cell cycle with subsequent synchronous transition to S-phase. An increased number of replicative forks simulated fragmentation of DNA (Pulito *et al.,* 1983). Some research teams did not detect any increase in DNA fragmentation in this model of differentiation (Sugiura *et al.,* 1984 Reboulleau *et al.*, 1983). At the same time an increased number of double-strand breaks was found in differentiating K562 erythroleukemia cells; these retained a high potential for DNA repair (Tabocchini *et al.,* 2000).

Lympohocytes

Quiescent lymphocytes contain more DNA strand breaks when compared to other human cells (Johnstone, Williams, 1982). Blast-transformation of lymphocytes was followed by an active DNA break repair; the process of blast-transformation was blocked by poly-ADP-ribosyltransferase inhibitors. In an analogous study conducted on murine lymphocytes the number of repaired DNA strand breaks was evaluated quantitatively, it was concluded that 2500 DNA strand breaks were repaired in every cell undergoing blast-transformation (Kaplan *et al.,* 1987; Johnstone, 1984). Stimulation of mouse spleen cells with Concavalin A triggers repair of 3200 DNA strand breaks per cell. Repair was observed both in T- and B-splenocytes, thymocytes contained a lesser number of DNA strand breaks that were not repaired during the blast-transformation. Repair was preceded by an increase of the intracellular NAD^+ concentration and increase of enzymatic activity of poly-ADP-ribosyltransferase. However, induction of the poly-ADP-ribose synthesis by injection of NAD^+ in permebialized cells did not cause blast-transformation. It was concluded that DNA repair is necessary, but insufficient for the blast-transformation (Greer, Kaplan, 1983; 1984; 1986). DNA repair in blast-transformed splenocytes is triggered by an increase in the intracellular Na^+ concentration (Prasad *et al.,* 1987). It was also shown that the DNA of quiescent lymphocytes compared to the DNA of blast-transformed cells contains a larger fraction of 50 Kbp DNA revealed by pulse-field electrophoresis, this indicates an increased number of double-strand breaks in quiescent cells (Szabo, Bacso, 1996). Repair of DNA strand breaks was observed also during stimulation of lympholeucemic cells. The high initial level of DNA strand breaks in these cells was attributed to low activity of DNA ligase (Feon *et al.,* 1988: Rusquet *et al.,* 1988). It should be mentioned that some teams could not reproduce the above data (Boerrigter *et al.,* 1989; Jostes *et al.,* 1989). The cause of the discrepancy was scrupulously studied by E. Moskaleva (Moskaleva, 1989; 1990; Moskaleva *et al.,* 1989), who revealed a more complicated course of events. It turned out that native cells circulating in blood do not contain many DNA strand breaks, the breaks accumulate during cultivation of the cells *in vitro*, however this team confirmed important DNA repair during some stages of the lymphocyte stimulation. Other authors claim that lymphocytes still contain more DNA strand breaks as compared to monocytes (Holz *et al.,* 1995). Theoretically, DNA breakage is necessary for the process of diversification of immunoglobulin genes. According to the modern point of view (Maizels, 2005) this process starts with expression of the activation-induced deaminase (AID). The enzyme deaminates the cytosine residues in specific G-rich sites of immunoglobulin genes (S-sites) that form single-strand hairpins during transcription. Deaminated bases are removed by DNA repair enzymes; the single-strand breaks arising due to removal of these bases facilitate the following recombination. It is considered

that non-immunoglobulin genes are protected against deamination by AID as these do not contain S-sites, although the possibility of deamination by other genes cannot be completely excluded. For example, there are indications of susceptibility of c-myc gene to AID action (Maizels, 2005). If it is the case that deaminase indeed affects the whole lymphocyte genome, the accumulation of DNA strand breaks described above could be explained by incomplete repair of deaminated sites, moreover when alkaline denaturation of DNA is used in protocols of DNA break detection, the deaminated alkali-labile sites can be taken for DNA strand breaks. The question merits a thorough study.

Myelocytes and other white blood cells

Stimulation of the bone marrow with granulocyte-macrophage stimulating activity is followed by formation and repair of DNA strand breaks (Francis *et al.,* 1984). A detailed study of the phenomenon showed that inducers of monocyte differentiation trigger repair of pre-existing DNA strand breaks, but inducers of granulocyte differentiation trigger both formation and repair of DNA strand breaks (Khan, Francis, 1987). Induction of differentiation of promyelocytic HL-60 cells is also followed by induction and repair of DNA strand breaks (Farzaneh *et al.,* 1987a, b). The loss of amplified *c-myc* sequences in differentiating HL-60 cells (Shima *et al.,* 1989) could be enabled by the above DNA strand breaks. Specific protein MGI-2 with nuclease activity is involved in granulocyte and macrophage differentiation (Weisinger *et al.,* 1986). Moreover differentiation of HL-60 cells is followed by increased expression of DNA-dependent proteinkinase involved in the repair of double-strand DNA strand breaks (Sallmyr *et al.,* 2004); expression of DNase II that produces above breaks is increased at the same time (Chou *et al.,* 2003).

Epidermal cells

DNA strand breaks were detected in the cells of intestinal epithelium, keratinizing epithelium and epithelium of salivary ducts (Iseki, 1986), terminally differentiated vaginal epithelium (Modak, Traurig, 1972) and during differentiation of keratynocytes in culture (Hartley *et al.,* 1985). Kidney and epididymys epithelium cell DNA contains a high level of DNA strand breaks (Fairbairn *et al.,* 1994). Cells of human lower lip salivary gland ductal epithelium contain numerous gaps revealed by filling-in by Klenow fragment, these cells also constitutively express Ku protein, this fact indicates intensive double-strand DNA break repair in these cells (Larsson *et al.,* 2001).

Plant tissues

Accumulation of double-strand DNA breaks was observed in developing barley shoots and during senescence of the leaves of barley and wheat plants (Chen, Srivastava, 1986; Sjakste *et al.,* 1990; 1993). Breakage of DNA in flower buds becomes more pronounced during breaking of dormancy and development of apple-tree buds (Li *et al.,* 1989). Mutation of TEB gene causing constitutive expression of genes involved in double-strand break repair is followed by numerous defects of *Arabidopsis* development. The amount of homologous recombination is decreased in these plants. These facts indicate the necessity for double-strand break induction in normal higher plant development (Inagaki *et al.,* 2006).

Possible mechanisms of DNA break formation in differentiating cells

Recombination and enucleation

The data presented above provide evidence for an association between differentiation and accumulation of DNA strand breaks. It is unclear however, how these breaks might be involved in the differentiation process. The generally accepted view claims that diminution of DNA and DNA recombination (the former is not possible without induction of DNA strand breaks and their repair) are minor mechanisms of gene activity regulation characteristic of very specialized cells and peculiar organisms. The DNA loss happens during enucleation of maturating mammal erythrocytes and lens cell differentiation, in the course of development of some protozoans, worms and crustaceans. Recombination is observed during the life cycle of yeast, antigen variation in trypanosomes and antibody production in higher organisms (Latchmann, 1995; De Maria *et al.,* 2004; Lieber, 1998). Actually, the process of induction of DNA double-strand breaks and their repair during meiotic recombination in yeast is known in detail and the enzymes that induce DNA strand breaks are also well-characterized (Sollier *et al.,* 2004). DNA sequences where these incisions are made (Klein *et al.,* 2005) have been characterized. Among the above examples recombination could explain the sometimes observed DNA repair

in blast transformed lymphocytes; however it seems doubtful that 2500 breaks per cell (Kaplan *et al.,* 1987) are needed to perform recombination in a single gene. It was speculated that numerous breaks indicated involvement of recombination in the differentiation process more frequent than generally assumed (Williams, Johnstone, 1983); however this hypothesis has been not proved in the succeeding twenty years. Recombination might be quite intensive also during neuron differentiation (Chun and Schatz, 1999; Gilmore *et al.,* 2000), but direct evidence for this has not been published yet.

Excision of modified bases

The above described deamination of cytosine in lymphocytes (Maizels, 2005) could explain the effect if involvement of vast genomic areas in the process is proved. It was also hypothesized that breaks in differentiated cells accumulate in the sites with demethylated bases (Patkin, 2002). Recently formation of DNA strand breaks in the promoter of the tyrosine amino transferase (*Tat*) gene in cultured hepatocytes was observed following the hormone-induced demethylation of cytosine residues in this gene (Kress *et al.,* 2006). Demethylation of the numerous genes necessary for execution of the differentiation program, during transcription outburst could lead to massive accumulation of DNA strand breaks; however this assumption should be tested experimentally. Mechanisms of DNA strand break formation are schematically presented in Figure 2.

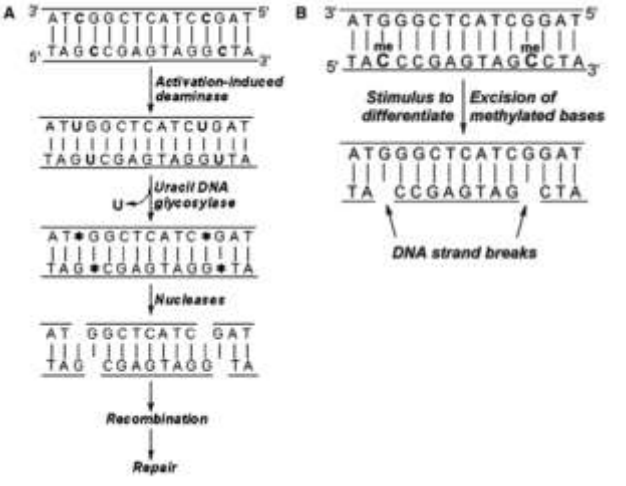

Figure 2. Schematic presentation of mechanisms of DNA strand break formation during deamination of cytosine (A) and excision of methylated cytosine (B).

DNA repair and differentiation

Decreased capacity of differentiated cells for DNA repair could explain accumulation of DNA strand breaks in such cells. In this case the cells would not be able to repair spontaneous DNA strand breaks (Vilenchik, Knudson, 2000; 2003). It is considered that insufficient DNA repair causes accumulation of DNA strand breaks in cells of aging organisms and senescent cell cultures (Bohr, 2002). Summarizing the results of numerous studies on repair intensity in differentiated cells performed in P. Hanawalt's and other laboratories, Nouspikel and Hanawalt (2002) come to the conclusion that DNA repair capacity is decreased in general during differentiation of neurons, muscle cells, adipocytes, white blood cells, keratonocytes, melanocytes, intestinal epithelium cells, hepatocytes , spermatids and oocytes. This conclusion is valid for nucleotide excision repair and repair of single-strand DNA breaks. In their most recent reports these authors provide evidence for the existence of a specific differentiation-associated type of repair active in terminally differentiated cells. This mechanism enables repair of DNA lesions inside chromatin domain of the genes that are expressed in a given type of differentiated cells. DNA lesions accumulate in the resting dormant part of the genome (Hsu *et al.,* 2007; Nouspikel *et al.,* 2006). Data supporting the idea of DNA repair decay in differentiated cells were obtained also on granulocytes differentiated from HL-60 cells. It was shown that the Ku protein, necessary for repair of double-strand breaks, is degraded by a specific protease (Sallmyr *et al.,* 2004). The authors explain their finding as a step of down-regulation of DNA repair capacity necessary for normal performance of granulocyte functions. They speculate that a high level of exposition of granulocytes to free radicals inevitably causes massive DNA damage. Repair of these lesions is not profitable from the point of view of ATP consumption, thus DNA repair systems are degraded in order to economize ATP. DNA remains unrepaired as a sequence. This could be a very logical explanation of the phenomenon of accumulation of DNA strand breaks in differentiating HL-60 cells at least. Decreased DNA repair is observed also in differentiated root cells in barley (Shikazono *et al.,* 1995). However, a decrease in the capacity for DNA repair cannot be ruled out as a general feature of differentiated cells. Other

groups find that DNA of differentiated cells is more resistant to DNA lesions produced by reactive oxygen species compared to undifferentiated cells (Covacci *et al.,* 2001); there are also reports indicating efficient DNA repair in such cells (Farzaneh *et al.,* 1987a). Fibrocytes derived from human fibroblasts also maintain efficient DNA repair systems (Brammer *et al.,* 2004). Thus, decreased DNA repair capacity cannot explain all cases of DNA break accumulation in differentiated cells.

Differentiation and poly(ADP)ribosylation

It was proposed that DNA strand breaks in differentiating cells arise as the necessary triggering factor for poly (ADP) ribose synthesis, as the subsequent changes in gene expression profile are regulated via poly(ADP)ribosylation of the chromatin proteins (Shall, 1983). Moreover, it was speculated that DNA strand breaks are induced and repaired constantly following the principle of dynamic equilibrium (Farzaneh *et al.,* 1985). The importance of poly (ADP) ribosylation of nuclear proteins in the differentiation process is confirmed by numerous novel investigations. For example, the poly (ADP) ribosylation level is modified during differentiation of numerous cell lines. In some cases inhibitors of poly (ADP) ribosylation block differentiation (Faraone-Mennella, 2005). Thus, the suggestion of a fundamental role for programmed DNA damage merits further development. The necessity for DNA break-dependent poly (ADP) ribosylation was demonstrated for early embryo development, during differentiation of neurons and in differentiation induced by retinoic acid (Bürkle, 2006). In the process of apoptosis triggered by DNA damage, exhaustion of the NAD^+ pool for synthesis of the ADP-ribose polymer and the subsequent failure of cellular bioenergetics is one of the causes of cell death (Koh *et al.,* 2005). Interestingly, this possibility was predicted as long as 20 years ago as a consequence of a shift of dynamic equilibrium to excessive DNA fragmentation (Farzaneh *et al.,* 1985). Thus, as the similarities between apoptosis and differentiation processes become more evident, the question should be discussed considerably more thoroughly than heretofore.

Interfaces between differentiation and apoptosis

The idea about a possible *unfinished* apoptosis was proposed by Solov'yan and co-authors (1997) who observed long-range DNA fragmentation in several cell types. Recent data on the existence of some common pathways for programmed cell death and differentiation enable further development of this idea. The most convincing evidence for association of the two processes was obtained after description of the vital functions of caspases; it turned out that these enzymes are involved in the processes of normal differentiation in Drosophila (Kumar, 2004) and mammals (Launay *et al.,* 2005). In the latter case participation of caspases in differentiation is not restricted to cells that become enucleated in the course of differentiation. Caspases participate in processes of blast-transformation of lymphocytes, differentiation of megakaryocytes and epithelial cells (Launay *et al.,* 2005). For example, caspases 6, 3, 8 and c-FLIP are necessary for lymphocyte differentiation (Siegel, 2006). However, enucleation and apoptotic chromatin fragmentation are executed by different nucleases (Nagata *et al.,* 2005).

We have observed simultaneous differentiation and apoptosis in the culture of AEV-transformed chicken erythroblasts of HD3 line (Iarovaia *et al.,* 2001; Sjakste, Sjakste, 2004). These cells start hemoglobin synthesis when kept at 42°C in presence of a protein kinase C inhibitor. As demonstrated in Figure 3 the uninduced culture where alphaA-globin gene is abortively expressed in the nuclei contains a small fraction of apoptotic cells. Transport of alpha globin mRNA to cytoplasm and DNA fragmentation was observed in the cells induced to differentiate. We remind readers that these were avian erythroblasts, in which the nucleus remains intact during differentiation.

Genomic sites for preferential DNA cleavage during differentiation

The NM23 protein which is involved in regulation of differentiation and tumor transformation binds the *c-myc* gene promoter and makes a double-strand cut inside it (Postel, 1999). The recombination hotspots probably exist constitutively as a site of single-strand DNA that can be registered as a break (Szekevolgyi *et al.,* 2005). Cloning and sequencing of genomic sites adjacent to breaks splitting the human genome in 50 Kbp fragments in the absence of apoptosis enable the characterization of several predominant motifs; CCAGCCTGG and AAAAAAAACAAAA were the most common, and some formerly known repeats including Alu repeats were also found in these sequences. The

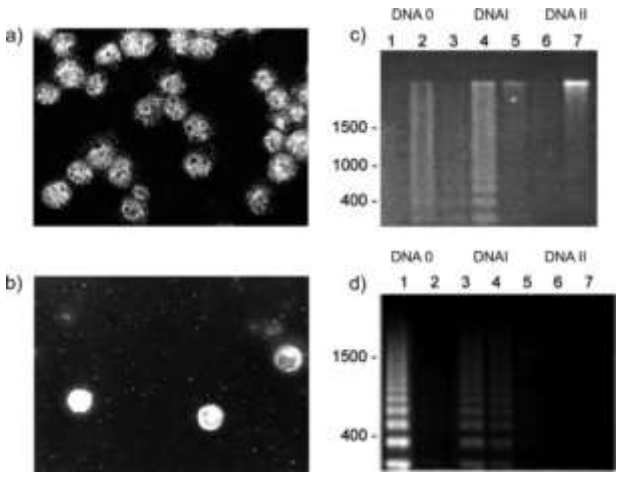

Figure 3. Simultaneous differentiation and apoptosis. *In situ* hybridization with alpha globin probe (a, b) and gel electrophoresis of DNA preliminary fractionated by means of nucleoprotein-celite chromatography. (c, d) of HD 3 cells. a, c – uninduced culture; b, d - culture induced to differentiate. DNA0 – fraction of chromatin unbound to the nuclear matrix, DNA I – fraction of chromatin loosely bound to the nuclear matrix, DNA II - fraction of chromatin tightly bound to the nuclear matrix. Lane 1 – breakthrough fraction; lanes 2,3 – fractions of NaCl gradient; 4,5 – LiCl-urea gradient; 6,7 – temperature gradient (see explanations below). Cell culture conditions and *in situ* hybridization protocol were as described (Iarovaia *et al.,* 2001). Principle and interpretation of nucleoprotein celite chromatography results are illustrated on Figure 5. Briefly the cell lysate was directly applied on a precooled (0°C) water-coated column of Celite R-630 (Fluka). Column was rinsed with 50 mL of 5 mM $MgCl_2$, 10 mM Tris HCl, pH 7.4 (breakthrough fraction). 80 mL of NaCl (0 - 2M) in linearly increasing concentration was pumped through the column; eluate was collected in two fractions (DNA 0). Then a gradient of LiCl - urea (0 - 4M; 8M) was applied in the same manner (DNA I). Finally the column was gradually heated from 0°C to 100°C under constant flow of 4 MLiCl, 8M urea solution (DNA II). Eluate obtained after each gradient was divided in two fractions: by volume in NaCl and LiCl-urea fradients, below 70°C and between 70°C and 100°C for the temperature gradient. Nucleic acids from the fractions were concentrated by absorption on hydroxyapatite and elution with 1 mL of 0.24 M phosphate buffer. DNA was purified by Wizard DNA Clean-Up System kit (Promega) and subjected to electrophoresis in 1% agarose in 1TBE buffer.

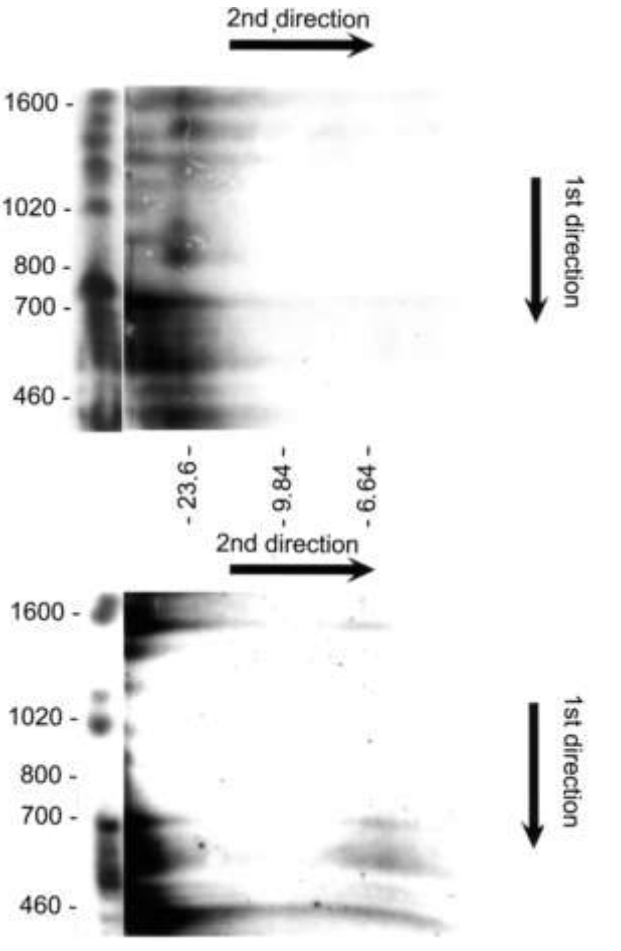

Figure 4. Individual peculiarities of long-range organization and localization of single-strand breaks in satellite 1 sequences of Xenopus erythrocytes. Upper and lower panels represent two animals. 1st direction - pulse-field electrophoresis of erythrocyte DNA digested with BamH1, 2nd direction – alkaline electrophoresis. Conditions of pulse-field electrophoresis and alkaline electrophoresis were as described for Figure 1c. Hybridziation with probe for satellite 1. Positions of the size markers (Kbp) are given on the left and between the panels. Details in (Sjakste, 1997).

authors suggest recombinogenic functions for these sequences (Szilagyi *et al.,* 2003). Similar recombinogenic functions are attributed also to the human 5 bp classical satellite $(TTCAA)_n$ and to similar mouse major satellite DNA. Using an original DNA-breakage detection – fluorescence *in situ* hybridization method (DBD-FISH) it was shown that these sequences contain numerous endogenous alkali-labile sites or DNA strand breaks in lymphoid cells (Fernandez *et al.,* 2001; Rivero *et al.,* 2001). The grasshopper *Pyrgomorpha conica* telomeric repeat $(TTAGG)_n$ in sperm cells also contains a number of endogenous DNA lesions (Lopez-Fernandez *et al.,* 2006). The Chinese Hamster Interstitial Telomeric Repeat Sequence also contains numerous endogenous single-stranded sites and DNA lesions in different strains of CHO (Chinese Hamster Ovary) cells (Rivero *et al.,* 2004).

Forum DNA is excised in the GGCTGGGCTGCCAA site and the $(TCAG)_{11}$ microsatellite is often found inside it. The authors indicate that these sites are situated not in the nuclear matrix attachment points but rather in special sites of heterochromatin intercalation (Tchurikov *et al.,* 1998; 2004). Evaluation of DNA breakage site-specificity in the *Xenopus* erythrocytes showed that the *fast* DNA fraction contained less rDNA and satellite 1 sequence compared to high molecular fraction. At the same time it was calculated that rDNA contains

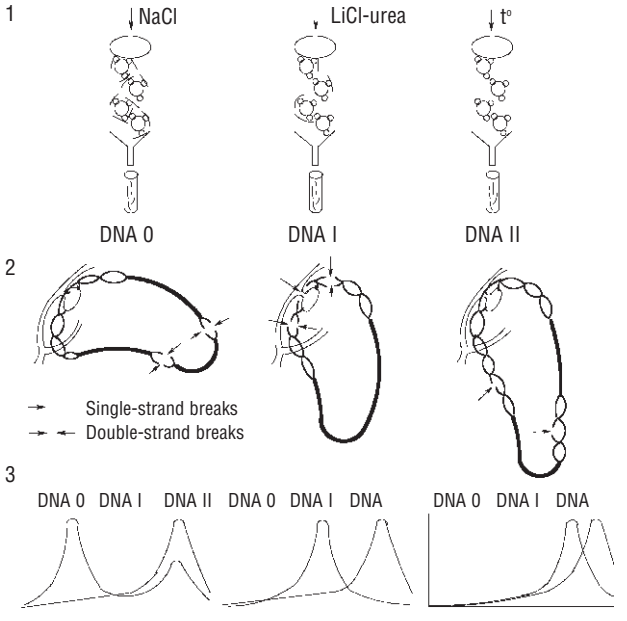

Figure 5. Principle and interpretation of results of the nucleoprotein celite chromatography method. 1. separation of factions; 2. possible localizations of DNA breaks; 3. corresponding changes in chromatograms. Single-strand breaks in distal parts of chromatin loop cause decrease of the DNA elution temperature. Single strand breaks in the vicinity of the replication complex induce transition DNAII – DNA I. Double strand breaks release the DNA0 fraction.

more DNA strand breaks (2 breaks per repeat) than satellite 1 (Sjakste, 1997). The distribution of DNA strand breaks inside satellite 1 was evaluated by means of two-dimensional pulse-field/alkaline electrophoresis. Results are presented in Figure 4. DNAs of two clawed toads were digested in agarose blocks with BamH1 restrictase and separated in the first direction by pulse-field electrophoresis. The gel slices were cut out and sealed in alkaline agarose gel. Electrophoresis in the 2nd direction was performed in conventional conditions for alkaline electrophoresis. DNA was blotted onto a membrane and hybridized with molecular probe for satellite 1. The first direction revealed individual differences in satellite 1 long-range organization (Pasero *et al.,* 1993). The second direction revealed discrete single-strand fragments about 20 Kb in size in the first animal (upper panel). This fraction was not observed in the second animal (lower panel), however a well-resolved fraction of about 4 Kb in size is clearly visible. Thus, the single-strand DNA strand breaks are specifically distributed in satellite 1 sequences of different individuals (Sjakste, 1997).

Finally, we would like to mention data obtained by means of a nucleoprotein celite chromatography method. The method is described in the legend to Figure 3. Its principle is illustrated in Figure 5. In numerous studies performed on animals (Lichtenstein *et al.,* 1982; 1995; Sjakste *et al.,* 1990) and plant cells (Sjakste *et al.,* 1993) we observed changes in chromatograms indicating induction of DNA strand breaks in quiescent or differentiating cells in close vicinity to the replication complex in matrix-attached DNA. Taken together the data about localization of differentiation-dependent DNA strand breaks indicate their selective accumulation in reiterated sequences and in sites of attachment to the nuclear matrix.

Conclusions

In this review we have made an attempt to summarize the available data on DNA strand breaks that accompany cell differentiation. Bad reproducibility of the results in different teams is the most disappointing discovery. Spermatide development is the only process in which accumulation of DNA strand breaks at a certain stage is a generally accepted fact. Data supporting formerly favorite models - blast-transformation of lymphocytes and myotube formation are rather contradictory. It seems that the changing fashion in detection methods for DNA strand breaks is one of the main causes of these discrepancies. The reviewed publications were issued during the past thirty years; several different methodical approaches were popular during different phases of this period. Principles of different methodical approaches of DNA break detection are summarized in Figure 6 and Figure 7. The oldest publications present mostly ultracentrifugation data, the eighties was the period of alkaline elution, alkaline unwinding and nick-translation, comet assay, while TUNEL-assay and pulse-field electrophoresis are the most popular since the mid-eighties till now. Should TUNEL-assay reproduce data obtained by alkaline unwinding? Not of course, as the former cannot detect DNA strand breaks without 3′-OH end, but the latter does not discriminate alkali-labile sites and DNA strand breaks. Going further we can mention that results of alkaline elution are influenced by the level of DNA superhelicity, features of the DNA-polymerase used determine to a large extent the results of the nick-translation, etc. The problem could be solved in a framework of a complex investigation in which several research teams follow the formation and repair of DNA strand breaks in several well-defined

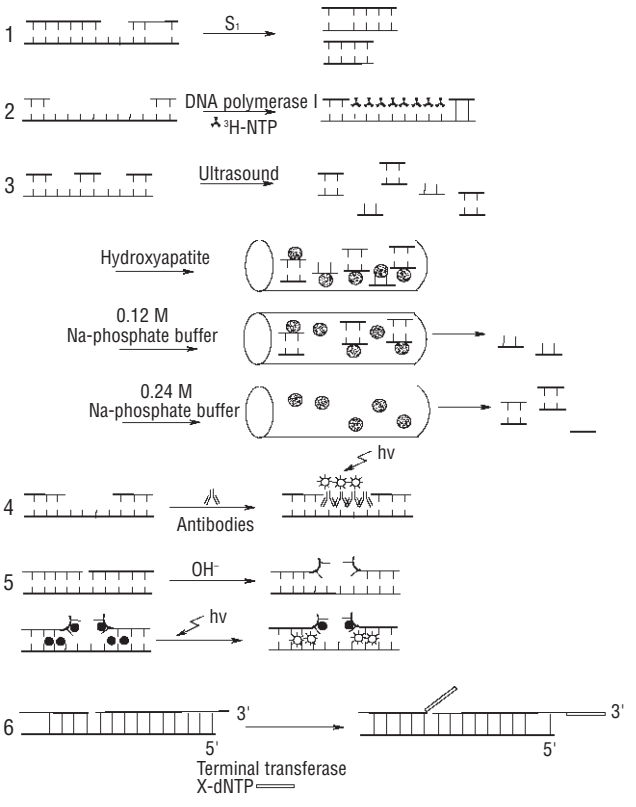

Figure 6. Methods of DNA break detection based on different features of single- and double-strand DNA. 1. elucidation of single-strand sites with S1-nuclease; 2. nick-translation; 3. alkaline unwinding on hydroxyapatite; 4. immune fluorescence method; 5. fluorometric analysis; 6. TUNEL assay.

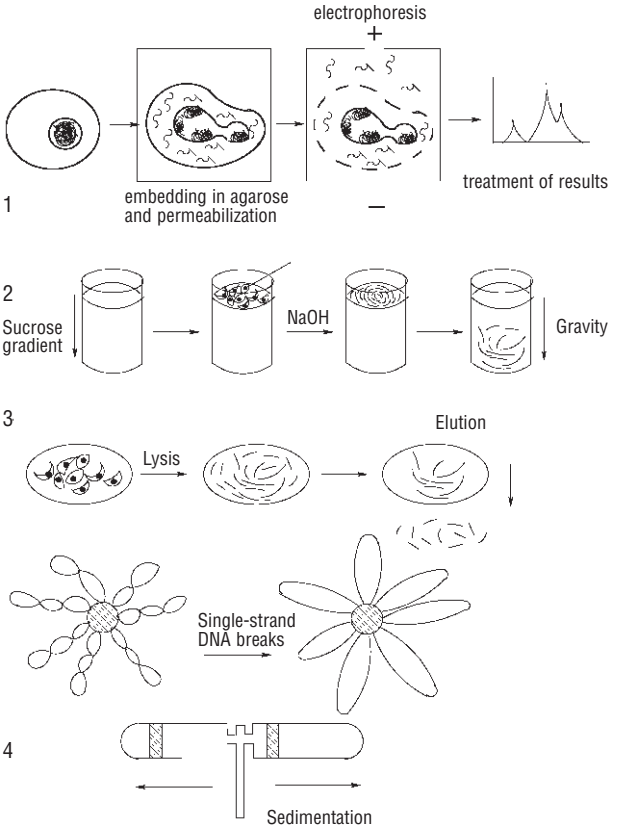

Figure 7. Methods of DNA break detection based on peculiarities of DNA fragments of different length and superhelicity changes. 1. comet assay; 2. sedimentation in sucrose gradient; 3. alkaline elution; 4. sedimentation of nucleoids.

models of differentiation (lymphocytes, myocytes, HL-60 cells) using set methods based on different principles. If break formation is confirmed, the structure of the breaks should be determined (double or single-strand breaks, breaks with free 3′-OH or 5′-OH ends, etc.) as this has never previously been performed.

Several hypotheses can be proposed to explain the possible functional role of DNA strand breaks in differentiation. The hypothesis proposed by Patkin (2002) about formation of the breaks in areas of base demethylation appears to be quite probable. The process can be imagined as follows: stimulus for differentiation triggers activation of formerly dormant genes; demethylation is followed by DNA break formation and repair. The process was well-illustrated on an example of the formation of DNA strand breaks in the promoter of the tyrosine amino transferase (Tat) gene in cultured hepatocytes following the hormone-induced demethylation of cytosine residues in this gene (Kress *et al.,* 2006).

It seems that gene recombination is more common in differentiation, including the neuron differentiation, than previously accepted (Chun, Schatz, 1999; Gilmore, 2000). Taking into account data on the recombination in lymphocytes (Maizels, 2005) the process can be imagined as follows: stimulus for differentiation is followed by deaminase expression, cytosine is deaminated in specific gene sites, excision of deaminated cytosine creates single-strand breaks transformed into double-strand breaks, and the latter enable the recombination process. Data on accumulation of DNA-breaks in recombinogenic sequences in (Szilagyi *et al.,* 2003; Fernandez *et al.,* 2001; Rivero *et al.,* 2001; Lopez-Fernandez *et al.,* 2006; Rivero *et al.,* 2004) confirm the possibility for frequent recombinations in different cells. If the recombination process is really wide-spread we should assume that different differentiated cells in

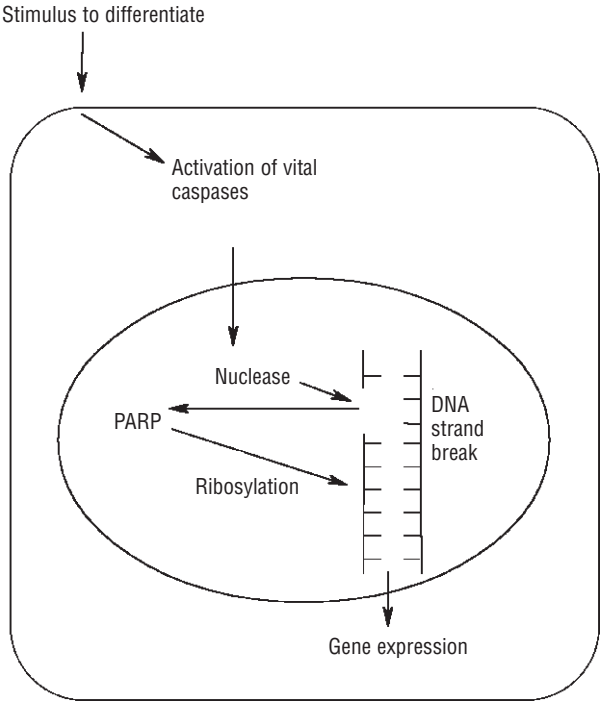

Figure 8. Hypothetical scheme of possible role of DNA breaks in differentiation with involvement of caspases, specific nuclease and poly(ADP)ribosyl transferase.

an organism should possess different genomes. We remind the reader that there is, in fact, nothing new in this postulate; it is well-known that cells differ in ploidy (haploid gametes, polyploidy hepatocytes). During the initial stages of enucleation the differentiating red blood cells or lens cells retain just a part of the genome. Lymphocytes differ among themselves and other cells in the structure of immunoglobulin genes. If the idea about intensive recombination in neurons is shown to be true (Chun and Schatz, 1999; Gilmore, 2000), each neuron should possess a unique genome! Perhaps the time has come to start projects in cell genomics? Modern methods enable comparison of the structure of a set of genes in individual cells of a given organism.

Finally, we can propose a hypothesis that assumes a more active role of DNA strand breaks in the differentiation process. It is based on the data on associations between differentiation and apoptosis processes, site-specificity of DNA strand breaks and activation of poly(ADP)ribose polymerase in differentiation. The cascade of events can be imagined as follows: stimulus for differentiation triggers activation of vital caspases, these activate a specific nuclease, the nuclease induces DNA strand breaks in specific sequences, poly(ADP)ribosyl transferase is activated in response, poly (ADP) ribosylation leads to expression of necessary genes (Figure 8). It should be mentioned that DNA strand breaks could cause changes in chromatin configuration favorable for expression of given genes. Although this hypothesis is still speculative, it does not contradict any well-established facts. In any case, an answer to the question about a functional role of DNA strand breaks in differentiation might be achieved if it attracts the interest of a broader research community.

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