Presence of Double-strand Breaks with Single-base 3' Overhangs in Cells Undergoing Apoptosis but Not Necrosis

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Abstract. Apoptotic cells in rat thymus were labeled in situ in paraffin-embedded and frozen tissue sections by ligation of double-stranded DNA fragments containing digoxigenin or Texas red. Two forms of double-stranded DNA fragments were prepared using the polymerase chain reaction: one was synthesized using *Taq* polymerase, which yields products with single-base 3' overhangs, and one using *Pfu* polymerase, which produces blunt-ended products. Both types of fragment could be ligated to apoptotic nuclei in thymus, indicating the presence in such nuclei of DNA double-strand breaks with single-base 3' overhangs as well as blunt ends. However, in nuclei with DNA damage resulting from a variety of nonapoptotic processes (necrosis, in vitro autolysis, peroxide damage, and heating) single-base 3' overhangs were either nondetectable or present at much lower concentrations than in apoptotic cells. Blunt DNA ends were present in such tissues, but at lower concentrations than in apoptotic cells. In contrast, in all of these forms of DNA damage, nuclei contained abundant 3'-hydroxyls accessible to labeling with terminal deoxynucleotidyl transferase. Thus, although single-base 3' overhangs and blunt ends are present in apoptotic nuclei, the specificity of the in situ ligation of 3'-overhang fragments to apoptotic nuclei indicates that apoptotic cells labeled in this way can readily be distinguished from cells with nonapoptotic DNA damage. These data are consistent with the involvement of an endonuclease similar to DNase I in apoptosis, which is predicted to leave short 3' overhangs as well as blunt ends in digestion of chromatin.

APOPTOSIS of mammalian cells is accompanied by cleavage of nuclear DNA within the linker regions between nucleosomes (2, 36). Double strand cleavage results from frequent nicks on both DNA strands (26). Although the endonuclease responsible has not been definitively identified, candidate nucleases with properties consistent with their involvement in apoptosis have been identified in apoptotic cells (17, 30, 39). These endonucleases are generally similar in their properties to pancreatic DNase I (21). Specifically, (*a*) the DNA ends produced by DNase I cleavage (5'-phosphate and 3'-hydroxyl) are the same as those found in apoptotic nuclei (1, 4, 25); (*b*) DNase I–transfected COS cells show chromatin changes similar to those seen in apoptosis (27); and (*c*) DNase I cleavage of chromatin produces the same characteristic nucleosomal DNA fragments that can be isolated from apoptotic cells (26). Although DNase I has been detected in cells undergoing apoptosis and the tissue distribution of DNase I is consistent with a role in apoptosis (28, 38), the endonucleases partially purified from apoptotic cells were

shown to be distinct from DNase I (17, 30, 39). There is less evidence for the involvement of DNase II and other endonucleases in apoptosis (12, 24).

In the experiments reported here, we determined whether DNA double-strand breaks characteristic of those produced by an endonuclease like DNase I can be detected in apoptotic cells in situ. When DNA is bound to histones or other proteins in chromatin, it is partially protected from the action of endonucleases, which are able to cleave the DNA at ~10-bp intervals, the distance of a single helical turn of the DNA (31). Because of the helical twist of DNA, the two strands are accessible to endonucleases with production of staggered ends as well as some blunt ends. Thus, DNase I cleavage of nucleosome-bound DNA gives rise to double strand cuts with 1, 2, or 3 bases of 3' overhang (7, 19). In contrast, DNAse II cleavage of DNA in chromatin yields longer 3' overhangs of an average of 4 bases (7, 31).

To detect double-stranded DNA ends in apoptotic nuclei in situ, we used two kinds of double-stranded labeled DNA fragments which were ligated to DNA ends in sections of fixed paraffin-embedded or frozen tissue samples. To detect single-base 3' overhangs, we took advantage of the fact that double-stranded DNA fragments synthesized by *Taq* DNA polymerase in the PCR have a single 3' base extension beyond the templated sequence. Although it

was originally suggested that *Taq* polymerase added only deoxyadenosine to the 3' ends of double-stranded DNA (6), other work subsequently established that if the last templated 3' nucleotide synthesized is deoxycytidine, *Taq* polymerase will add deoxyadenosine or deoxycytidine, leaving no blunt-ended DNA (15), thus providing a fragment that could potentially ligate to the recessed 5' base of many of the single-base 3' overhangs in a random DNA sequence. For comparison, a fragment ligatable only to blunt ends was synthesized by *Pfu* DNA polymerase, because this polymerase produces only blunt-ended products (15).

By this procedure we determined that apoptotic nuclei, but not nuclei in necrotic tissue or tissue with other non-apoptotic DNA damage, have DNA ends ligatable to labeled DNA fragments with single-base 3' overhangs. In contrast, nuclei with all forms of DNA damage have a high concentration of 3'-hydroxyl DNA ends that are a substrate for terminal deoxynucleotidyl transferase (TdT)[1] (11). TdT can extend the 3' base of single-stranded DNA and overhanging, blunt, and recessed 3' bases of double-stranded DNA (13).

Materials and Methods

Preparation of Double-strand DNA Fragments for In Situ Ligation

A 226-bp double-stranded DNA fragment was prepared using primers 5'-GTGGCCTGCCCAAGCTCTACCT-3' and 5'-GGCTGGTCTGCC-GCCGTTTTCGACCCTG-3' complementary to plasmid pBluescript-bSDI1 (37). Although we used this sequence for the data presented here, the actual sequence of the fragment used is unimportant because we used unrelated sequences of lengths 60–450 bp with equivalent results. To prepare fragments by the PCR with *Taq* polymerase, we set up reactions comprising 100 μl of 50 mM Tris-HCl, pH 8.3, 10 mM KCl, 1.5 mM MgCl₂, 16.6 μM digoxigenin-11-dUTP (Boehringer Mannheim Corp., Indianapolis, IN), 16.6 μM TTP, 50 μM dATP, 50 μM dCTP, 50 μM dGTP (other nucleotides from Sigma Chem. Co., St. Louis, MO), 100 pmol of each primer, and 10 pg of plasmid. For preparation of fluorescent DNA probes, 16.6 μM Texas red-12-dUTP (Molecular Probes, Eugene, OR) was substituted for digoxigenin-labeled dUTP. *Taq* polymerase (2.5 U, Boehringer Mannheim) was added to each tube when the reaction mixture was heated to 80°C. PCR was performed with 35 cycles of 20 s at 95°C, 20 s at 61°C, and 120 s at 74°C, the final cycle having an extension time of 4 min. Fragments were prepared using cloned *Pfu* polymerase (Stratagene, La Jolla, CA) using the same protocol but with a buffer composition of 200 mM Tris-HCl pH 8.8, 100 mM KCl, 100 mM ammonium sulfate, 20 mM MgSO₄, 1% Triton X-100 and 1 mg/ml BSA. Agarose gel electrophoresis of an aliquot of the reaction showed a single product for both enzymes. To precipitate the fragments, ammonium acetate was added to 2.5 M, the solution was centrifuged at 10,000 g for 5 min, and the supernatant was mixed with 2 vol ethanol and centrifuged for 25 min. The supernatant was discarded and the pellet was washed with 70% ethanol, and then with 100% ethanol. After vacuum drying for 20 min, the pellet was dissolved in water and the concentration measured by HOECHST dye 33258 fluorescence (3). The fragments were stored at −20°C until use.

In Situ Ligation of Labeled DNA Fragments on Tissue Sections

Digoxigenin- or Texas red–labeled fragments were ligated to DNA in tissue sections in situ using T4 DNA ligase. Various tissues (described in Results) were used with the following protocol. Tissue fragments were fixed in either freshly prepared paraformaldehyde or buffered formaldehyde, with equivalent results, and were conventionally dehydrated and embedded in paraffin. 6-μm sections were deparaffinized with xylene and rehy-

1. *Abbreviation used in this paper:* TdT, terminal deoxynucleotidyl transferase.

drated in graded alcohol concentrations. In some experiments, tissue was frozen in 2-methylbutanol in liquid nitrogen, embedded in Cryomatrix (Shandon Lipshaw, Pittsburgh, PA) and 5-μm sections were cut using a cryostat. Sections were fixed in cold acetone.

All the following procedures were performed at room temperature (23°C). Paraffin sections, but not frozen sections, were incubated with 50 μg/ml proteinase K (Sigma) in PBS for 30 min, and then rinsed thoroughly with water. A mix of 50 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA, 15% polyethylene glycol (8,000 mol wt, Sigma), with the digoxigenin- or Texas red–labeled DNA fragment at 1 μg/ml and DNA T4 ligase (Boehringer Mannheim) at 25 U/ml was added (20 μl per section). Sections were covered with glass coverslips and placed in a humidified box for 1 h. The sections were thoroughly washed in 70°C water. Sections labeled with fluorescent DNA fragments were observed immediately, optionally after counterstaining with 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI).

For digoxigenin-labeled fragments, sections were preblocked with blocking solution (Boehringer Mannheim), reconstituted as recommended by the manufacturer, for 15 min. The blocking solution was removed and sheep anti-digoxigenin Fab fragment-alkaline phosphatase conjugate (Boehringer Mannheim), 1:100 dilution in blocking buffer, was added for 10 min, followed by washing in 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, two times for 10 min each. For color development, sections were then placed in the solution recommended by the manufacturer (0.1 M Tris, pH 9.5; 0.1 M NaCl; 167 μg/ml 5-bromo-4-chloro-3-indolyl phosphate; 330 μg/ml nitro blue tetrazolium) and the color development was monitored under the microscope. The reaction was stopped by washing sections in water. The wet sections were photographed without counterstain.

Terminal Deoxynucleotidyl Transferase

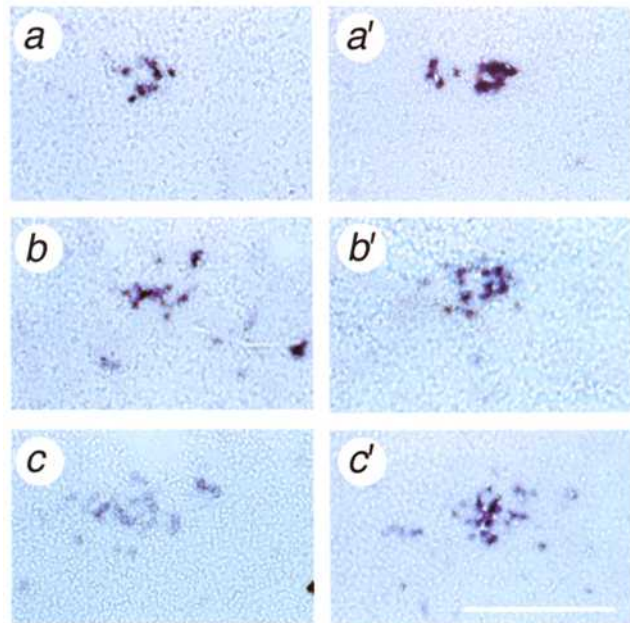For the reaction of available DNA 3' hydroxyls with TdT, the published

Figure 1. Detection of different types of DNA ends within apoptotic cells in rat thymus. Rat thymus was fixed and processed by three labeling methods as described in the text. The figure shows the reaction products resulting from (*a*) ligation of single-base 3' overhang double-stranded DNA fragment prepared by *Taq* polymerase, using 15 min of alkaline phosphatase color development; (*b*) ligation of blunt-ended DNA fragment prepared by *Pfu* polymerase, using 15 min of alkaline phosphatase color development; (*c*) extension of 3' hydroxyls with TdT, using 7 min of alkaline phosphatase color development. *a'*, *b'*, and *c'* are the reaction products of the three labeling methods using sixfold longer times of alkaline phosphatase color development (90 and 42 min, respectively). Bar, 200 μm.
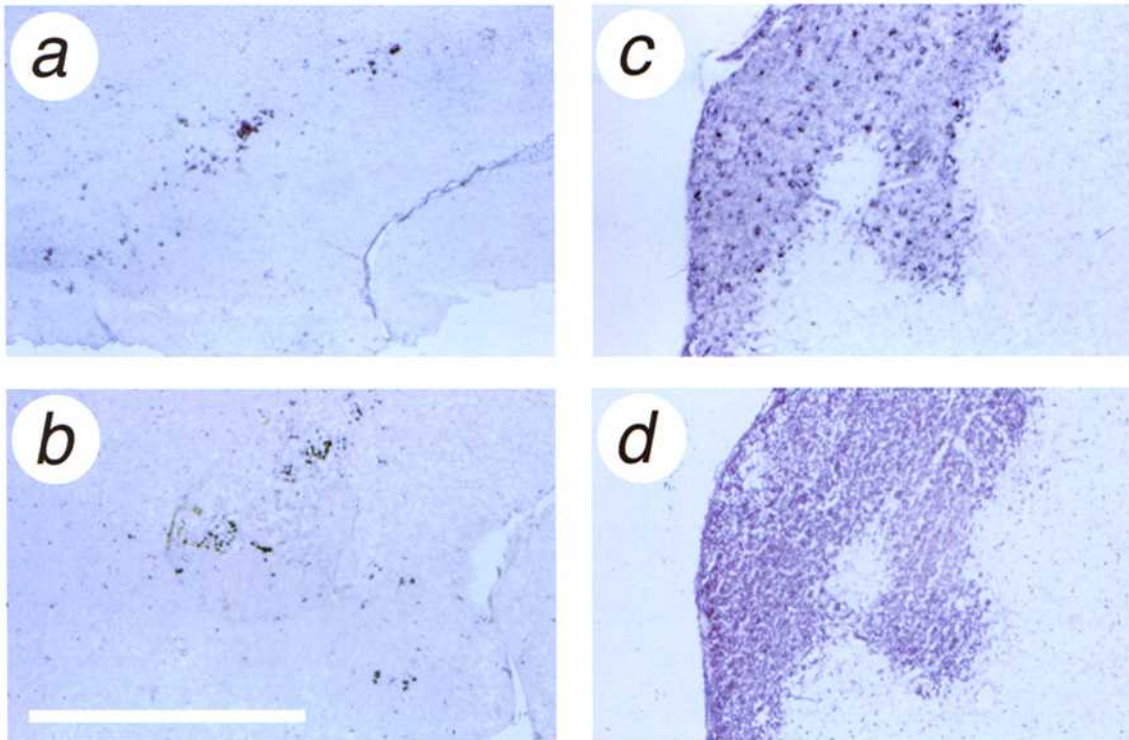
Figure 2. Comparison of patterns of apoptotic cells, detected by the presence of different types of DNA ends, in control and glucocorticoid-treated thymus. Thymus from control (*a* and *b*) and glucocorticoid-treated (*c* and *d*) rats were fixed and processed as described in Fig. 1. The figure shows the reaction products resulting from (*a* and *c*) ligation of *Taq* polymerase fragment, 15 min of alkaline phosphatase reaction; (*b* and *d*) TdT reaction, 7 min of alkaline phosphatase reaction. Bar, 2 mm.

procedure (11) was modified to accommodate the use of digoxigenin as a label rather than biotin. Instead of addition of ligase mixture as above, a mixture comprising 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.1 mM DTT, 50 μM digoxigenin-dUTP and 300 U/ml TdT (Promega, Madison, WI) (20 μl per section) was added for 1 h at 37°C in a humidified incubator. The washing and visualization of incorporated digoxigenin was as described above.

Tissues

Thymus. Sprague-Dawley rats (150 g) were injected subcutaneously with 6 mg/kg dexamethasone (Sigma) dissolved in 30% dimethyl sulfoxide in water. Animals were killed after 24 h and the thymus was either frozen as described above or fixed in 4% paraformaldehyde. Thymus from control animals was obtained and fixed in the same way. After 18 h in paraformaldehyde the tissue fragments were placed in 70% ethanol and taken through graded alcohols to 100% ethanol, placed overnight in chloroform, and then embedded in paraffin.

Necrotic Tissue. Sections from a Wilms' tumor from a 5-yr-old male patient, containing extensive areas of necrosis, as often encountered in such tumors (10), was used as to provide sections with large numbers of necrotic cells.

Hydrogen Peroxide-treated Liver. Sprague-Dawley rats were anesthetized and 100 μl of 30% hydrogen peroxide was injected superficially in the liver in several locations. After 20 s the liver was excised from the animal and the hydrogen peroxide–treated segment of the liver was fixed in 4% paraformaldehyde, with multiple changes of the solution to remove any remaining hydrogen peroxide. Segments of tissue from a distal region of the liver were fixed as controls. The tissues were then processed as described for thymus.

Tissue for In Vitro Autolysis. 5-mm fragments of bovine adrenal gland were placed in culture medium in a 37°C incubator for 16 h (9). They were then fixed and processed as described for thymus.

Heated Tissue Sections. Sections from a control bovine adrenal gland were deparaffinized, rehydrated through graded alcohol concentrations, placed in 0.1 M sodium citrate, pH 6.0, and heated at 100°C for 5 min.

Spotting of Digoxigenin-labeled DNA on Nylon

Different amounts of digoxigenin-labeled DNA (synthesized by random-primer method, Boehringer Mannheim) were spotted on Hybond-N membranes (Amersham Corp., Arlington Heights, IL) as previously described (8). The digoxigenin fixed to the membranes was detected using the protocol described above for tissue sections, except that anti–digoxigenin-alkaline phosphatase conjugate was used at 1:5,000 dilution.

Results

Detection of High Concentrations of Double-stranded DNA with Single-base 3′ Overhangs, as Well as Blunt-ended DNA and Free 3′ Ends in Apoptotic Cells

To obtain a tissue with many apoptotic cells we initially used rat thymus 24 h after administration of glucocorticoid, a model for apoptosis well established by previous investigators (36). However, control rat thymus also had a lower but useful number of apoptotic cells, consistent with the observation that ~1% of thymic cells show features of cell death in postnatal animals (23). Because the extensive cell death in the severely atrophic thymus of glucocorticoid-treated animals interfered with the identification of individual nuclei when using alkaline phosphatase detection, we used the apoptotic cells in control thymus as our standard for the investigation of DNA ends, but we show also for comparison the results in thymus from dexamethasone-treated animals.

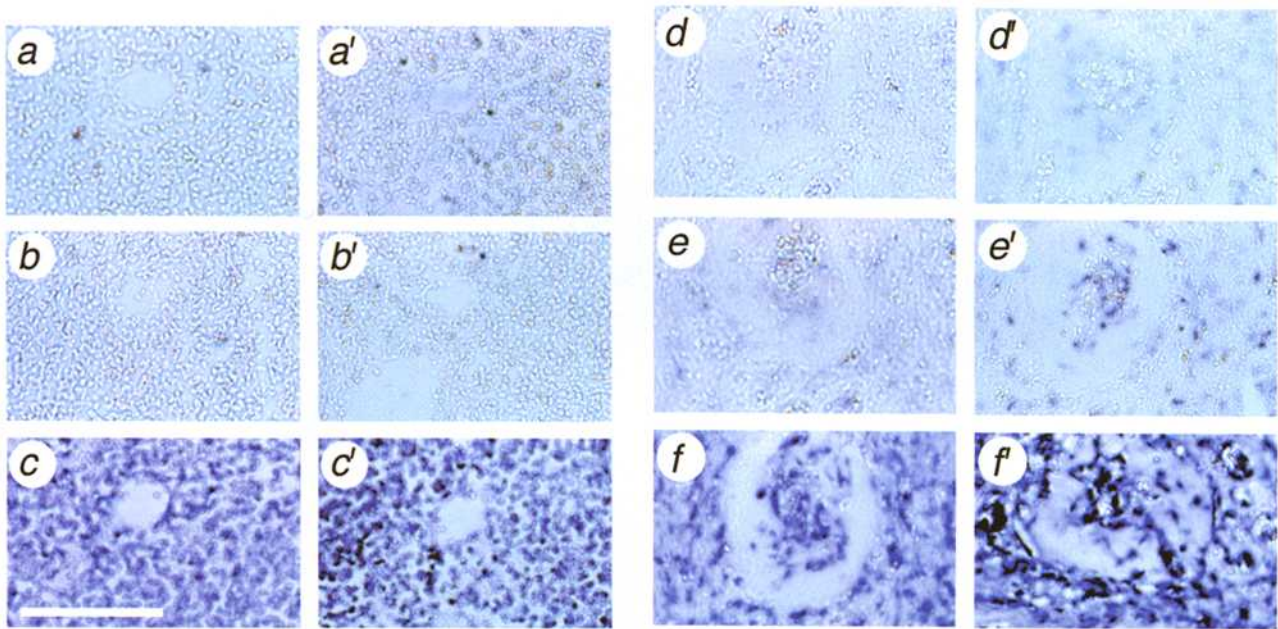Consecutive 6-μm sections were labeled with single-base 3′ overhang and blunt-ended DNA fragments, using

Figure 3. Detection of DNA ends within necrotic cells in Wilms' tumor by three labeling methods. The methods used were as described in Fig. 1. Serial sections were used and the same regions are shown by the three labeling methods. (*a* and *d*) Ligation of *Taq* polymerase fragment, 15 min of alkaline phosphatase reaction; (*b* and *e*) ligation of *Pfu* polymerase fragment, 15 min of alkaline phosphatase reaction; (*c* and *f*) TdT reaction, 7 min of alkaline phosphatase reaction. *a'*, *b'*, *c'*, *d'*, *e'*, and *f'* are the reaction products of the three labeling methods using sixfold longer times of reaction with alkaline phosphatase (90 and 42 min, respectively). Bar, 200 μm.

the same concentration of fragment and the same period of time of incubation with ligase. Consecutive sections were also labeled with TdT. In all cases the digoxigenin fixed to the section by the action of ligase or TdT was detected using an anti-digoxigenin–alkaline phosphatase conjugate. We assessed the length of time of the color development in the alkaline phosphatase reaction which just allowed the visualization of apoptotic cells. A reaction time of 7 min in the case of TdT and 15 min for ligase (using either 3'-overhang or blunt-ended DNA fragments) was sufficient to label apoptotic cells in thymus sections. When sixfold longer times of color development were used, the same number of nuclei were labeled, using all three techniques, with very little background staining (Fig. 1).

We compared the labeling of cells by ligation of 3'-overhang fragments with labeling of accessible 3' ends, in both control and glucocorticoid-treated thymus (Fig. 2). The numbers and patterns of cells stained by both methods in thymic cortex in control and glucocorticoid-treated animals were consistent with the previously reported numbers and patterns of apoptotic cells in control animals and in animals 24 h after glucocorticoid administration (23, 33, 34).

Comparison of Apoptotic and Necrotic Cells: Single-base 3' Overhangs Are Specific for Apoptotic Cells

To compare the occurrence of single-base 3' overhangs, blunt ends, and all accessible 3' ends in apoptotic and necrotic cells, we performed simultaneous staining of both apoptotic and necrotic tissue. Sections of Wilms' tumor, containing large areas of necrosis, were used as a standard for a tissue comprising many necrotic cells. Consecutive

sections were used to enable comparison of the reaction of different methods on various areas within this heterogeneous tissue. Staining reactions were performed for two different times, as used in the detection of apoptotic cells in thymus: a time sufficient for visualization of apoptotic cells (7 or 15 min) and a time sixfold longer. To ensure that these times were appropriate, sections of rat thymus were mounted on the slide together with the necrotic tissue and processed in the same labeling solutions. In necrotic areas of the specimen, the TdT reaction produced intense labeling even with 7 min of alkaline phosphatase color development (Fig. 3). In purely necrotic regions (Fig. 3, *a–c*), ligation of both 3'-overhang and blunt-ended fragments produced very little staining even with sixfold longer color development than required for visualization of apoptotic cells. In adjacent areas of the tumor, where some tissue structure was preserved (Fig. 3, *d–f*), extensive staining of nuclei with TdT was again observed. Distinct labeling of nuclei was also observed with sixfold overde-
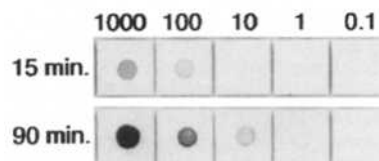

Figure 4. Relative color development of spots with various amounts of digoxigenin-labeled DNA. The indicated amounts (in pg) of digoxigenin-labeled DNA were spotted onto nylon membranes as described in Materials and Methods and then processed for detection of digoxigenin by the alkaline phosphatase reaction. The extent of color development at 15 and 90 min is shown.
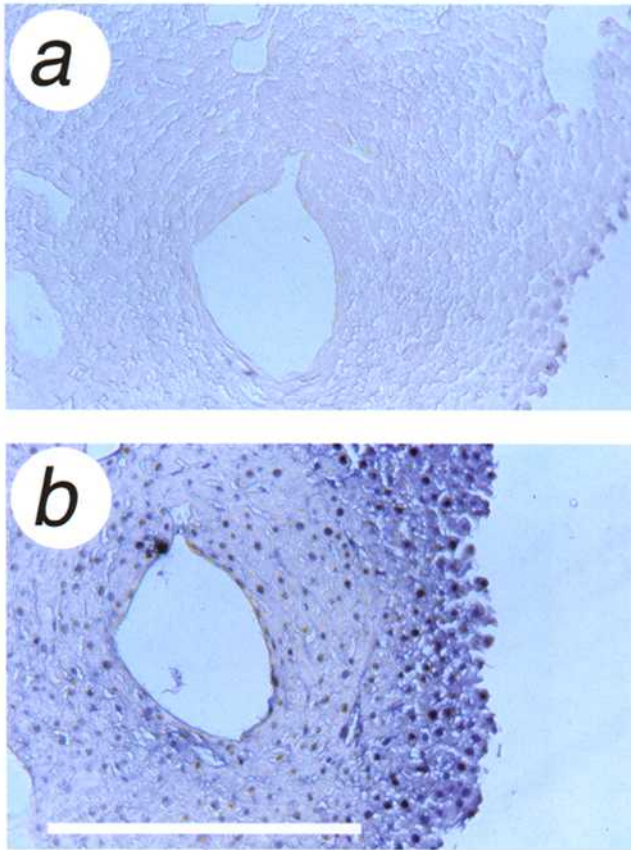
Figure 5. Detection of DNA ends within hydrogen peroxide–treated liver by two labeling methods. The methods used were as described in Fig. 1. The figure shows serial sections using (*a*) ligation of *Taq* polymerase fragment, 90 min of alkaline phosphatase reaction; (*b*) TdT reaction, 42 min of alkaline phosphatase reaction. Bar, 800 μm.

Figure 6. Detection of DNA ends within autolytic bovine adrenocortical tissue (see Materials and Methods). The methods used were as described in Fig. 1. Serial sections were used and the same regions are shown by the three labeling methods. (*a*) Ligation of *Taq* polymerase fragment, 15 min of alkaline phosphatase reaction; (*b*) ligation of *Pfu* polymerase fragment, 15 min of alkaline phosphatase reaction; (*c*) TdT reaction, 7 min of alkaline phosphatase reaction. *a'*, *b'*, and *c'* are the reaction products of the three labeling methods using sixfold longer times of reaction with alkaline phosphatase (90 and 42 min, respectively). Bar, 200 μm.

velopment of the color reaction in the case of the blunt-ended fragment, but hardly at all with the 3'-overhang fragment.

To provide an estimate of the relative abundance of single-base 3' overhangs in apoptotic vs necrotic tissue, a series of spots of digoxigenin-labeled DNA on nylon was stained by alkaline phosphatase color development using the same two times used for tissue sections (Fig. 4). This experiment shows that 90 min vs 15 min of color development allows the visualization of almost 100-fold less digoxigenin. Since apoptotic nuclei in thymus are readily detectable by ligation of 3'-overhang fragment using 15 min of color development, but nuclei in necrotic tissue are hardly stained with 90 min of color development, it may be concluded that apoptotic nuclei have at least 100-fold more single-base 3' overhangs than necrotic nuclei.

Absence of Ligatable DNA Ends in Tissues with Other Forms of Non-Apoptotic DNA Damage

We tested whether DNA ends ligatable to 3'-overhang DNA fragment were present in cells with DNA damage that might be produced in vivo (e.g., oxygen free radical DNA 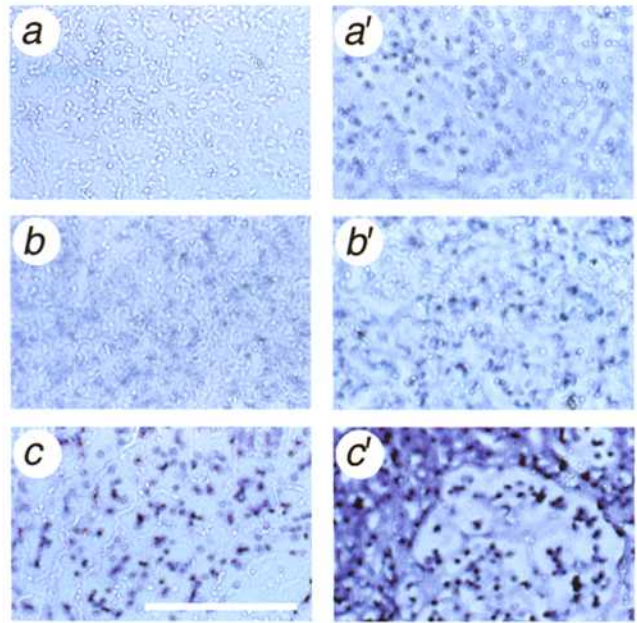strand breakage), or by postmortem autolysis, or by in vitro procedures that damage DNA (e.g., heating, as used in antigen retrieval procedures).

To provide rapid damage by oxygen radicals, at such a short time that apoptotic cell death was unlikely, hydrogen peroxide was injected into the liver of an anesthetized rat, and segments of liver were fixed 20 s later. This tissue showed many areas of nuclei with 3' ends accessible to TdT but no 3' overhangs (Fig. 5).

Autolysis was produced within the centers of fragments of bovine adrenal cortex incubated in medium at 37°C for 16 h (9). Again using times of alkaline phosphatase color development suitable for the detection of apoptotic cells within rat thymus, no cells were stained by ligation of 3'-overhang fragments, cells were lightly stained with blunt-end fragment ligation, and markedly stained by TdT extension of 3' ends (Fig. 6, *a–c*). With sixfold overdevelopment of the color reaction, light staining was apparent in the sections with 3'-overhang ligation whereas staining with blunt-end fragment and TdT became more intense (Fig. 6, *d–f*).

To test the effects of heat, 6-μm sections of control bovine adrenal gland were heated at 100°C for 5 min followed by ligation of 3'-overhang fragment or detection of accessible 3' ends (Fig. 7). Some nonspecific staining was noted in sections with ligation of 3'-overhang fragment but nuclei were not stained. In contrast, TdT labeling of nuclei was extensive in heated sections.
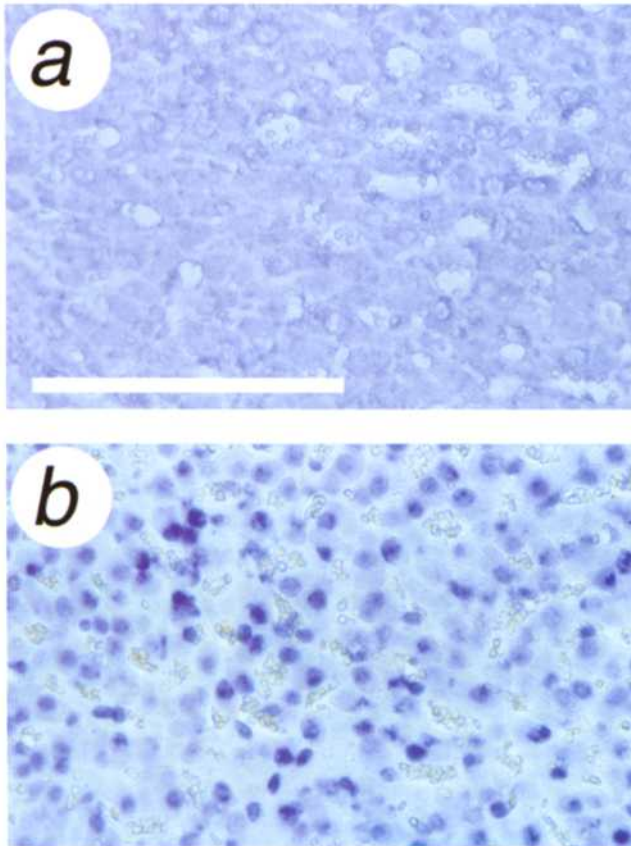
Figure 7. Detection of DNA ends within heated tissue sections by two labeling methods. Sections of bovine adrenal gland were treated as described in Materials and Methods. The figure shows serial sections using (*a*) ligation of *Taq* polymerase fragment, 15 min of alkaline phosphatase reaction; (*b*) TdT reaction, 7 min of alkaline phosphatase reaction. Bar, 200 μm.

Visualization of Ligatable DNA Ends in Thymus Using Fluorescent DNA Fragments

To clearly distinguish the morphology of nuclei labeled by ligation of 3′-overhang fragments, such fragments were labeled with Texas red rather than digoxigenin. Fig. 8 shows that the pattern of labeled cells in dexamethasone-treated thymus was identical to that observed with digoxigenin-labeled DNA(compare Fig. 2 *c*). However, individual nuclei were readily observable. In addition, ligation of fluorescent DNA fragment was shown to be applicable to labeling apoptotic cells in frozen sections of thymus. Essentially identical patterns of labeling were seen in both control and dexamethasone-treated thymus when frozen sections were compared to paraffin sections. Individual labeled cells in frozen sections showed nuclear changes characteristic of apoptosis (Fig. 8 *b*).

Discussion

We find that double-stranded DNA ends with 3′ single-base overhangs are characteristic of apoptotic cells. The presence of ligatable 5′ ends within a single-base recess implies a 5′ phosphate and some portion of ends with single-base 3′ overhangs; these features are characteristic of
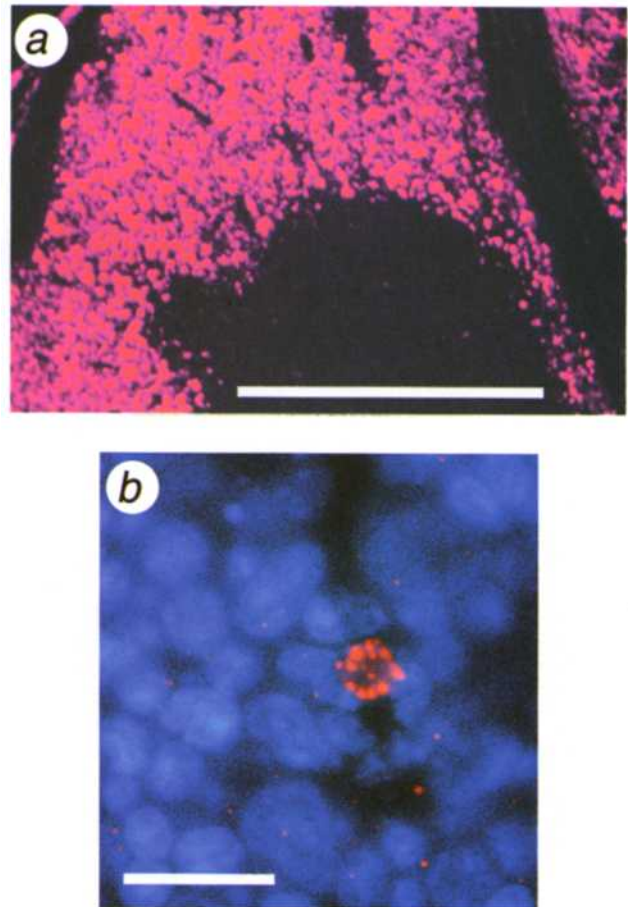
Figure 8. Detection of single-base 3′ overhang DNA ends in thymus using a fluorescent DNA fragment. (*a*) Section from the same glucocorticoid-treated rat thymus shown in Fig. 2 with ligation of Texas red–labeled DNA fragment. The section was not counterstained; the dark area in the center is the medulla, relatively unaffected by glucocorticoid treatment, with extensive cell death in the cortex. Bar, 800 μm. (*b*) Single Texas red–stained cell showing extensive nuclear fragmentation in frozen section of control thymus. Section was counterstained with DAPI to show surrounding intact nuclei. Bar, 80 μm.

the action of a DNAse I-type of endonuclease on chromatin. The primers used to prepare the ligatable DNA fragments were not treated with polynucleotide kinase to phosphorylate the 5′ base, and therefore the possibility of ligating the fragment to a 3′ hydroxyl on the end of a >1-base overhang, or to single-stranded DNA, or to the 3′ hydroxyl of a nick which has undergone partial denaturation, are eliminated. Additionally, T4 DNA ligase cannot join a 3′-phosphate to a 5′-hydroxyl (22), and therefore such ends, if present, do not contribute to the staining observed in apoptotic cells.

Random cleavage by an endonuclease producing multiple single strand nicks within internucleosomal DNA would not be expected to produce large numbers of single-base 3′ overhangs, which are predicted only when the DNA is protected by bound protein (7, 19, 31). However, apoptotic cleavage of DNA has been shown to produce an abundance of DNA fragments not only of the intranucleo-

somal length (~140 bp), but also of the length of the intra-nucleosomal DNA plus linker DNA (~195 bp) (26). Thus it is predicted that the endonuclease involved in apoptosis does not initially have access to the entire internucleosomal region, but preferentially to the sites at the junction of the nucleosome and the internucleosomal region. This may arise from the higher order structure of chromatin in the nucleus (35). The finding of single-base 3' overhangs thus is consistent with limited access of chromatin to the apoptotic endonuclease, the staggered cleavage resulting from the helical twist of the DNA (31).

Such overhangs were not detected in a direct test of the nature of the ends of apoptotic DNA fragments, in which no mobility shift of the fragments was found after treatment with T4 DNA polymerase in the presence of deoxyribonucleotide triphosphates (1). The presence of 3' overhangs would have been detected as increased mobility due to the exonuclease activity of T4 polymerase on such overhangs, and the presence of 5' overhangs would have been detected as decreased mobility due to the lengthening of the recessed 3' ends by polymerase activity. The absence of a mobility shift was interpreted as indicating that apoptotic DNA fragments are blunt-ended. However, the following considerations allow the conclusion that this experiment does not conclusively rule out the presence of 3' overhangs in apoptotic DNA. First, the removal of a single base would produce a very small change in mobility, which could easily be masked in a population of fragments with a range of lengths; second, there is no reason to suppose that all the apoptotic DNA fragments have the same ends, as it is clear from the present experiment that many are blunt-ended, thus more easily masking a shift due to single-base overhangs in a fraction of the molecules. Therefore, although apoptotic DNA clearly contains blunt ends, we think that the available data do not provide evidence against the presence of single-base overhangs in apoptotic DNA.

In the present experiments, single-base 3' overhangs were not found, or were present at <1% of the concentration found in apoptotic cells, in nuclei with DNA damage caused by a variety of means other than apoptosis. In all of these cases, the presence of 3' ends accessible to TdT was observed, apparently at concentrations at least as high as those in apoptotic nuclei. The presence of large numbers of accessible 3' ends in necrotic and autolytic tissue is predictable if during necrosis DNA is digested by a mixture of lysosomal nucleases (14, 32). The destruction of DNA in necrosis is thought to be a late rather than an early event (20). In this case, the bulk of the destruction of the DNA may occur when the chromatin is in a more relaxed form, and the supposed greater variety of enzymes involved in destruction of the cells may give rise to a more random pattern of DNA fragmentation. Nevertheless, it has been noted that necrosis can give rise to a nucleosome-length DNA ladder (5, 29). The presence of some blunt ends in necrotic and autolytic cells is presumably the result of the combined action of endonucleases followed by exonucleases that flush staggered double-strand fragments. The much lower level of 3' single-base overhangs may result from the probable transient nature of such ends, if produced, in the presence of a mixture of nucleases.

The presence of TdT-accessible 3' ends in heated sections of normal tissue may result from the denaturation of nicks already present in the chromatin as a result of ongoing DNA repair, or could result from the effects of heat per se (18). In any case, the production of accessible 3' ends in this way did not give rise to ends ligatable to 3'-overhang DNA fragments. Hydrogen peroxide produces large numbers of single-strand breaks in DNA in intact cells, which may have 3' hydroxyls, particularly following exonuclease action on the initial strand breaks formed (16). Again, these 3' ends, or some fraction of them, are accessible to TdT but are not ligatable to 3'-overhang DNA fragments.

The presence of single-base 3' overhangs in apoptotic nuclei is consistent with the involvement of a DNase I-type of endonuclease with restricted access to DNA in chromatin. The ligation procedure described here should provide a reliable and sensitive method for the detection of apoptotic nuclei with such ends, enabling discrimination of such cells from cells with other forms of DNA damage that yield accessible 3' DNA ends.

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