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Nonradioactive *in Situ* Nick Translation: A Useful Molecular Histochemical Tool to Detect Single-Stranded DNA Breaks

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At various stages of the life cycle of cells, the occurrence of DNA single-strand breaks (SSB) is a common event. In order to understand better the relationship of SSB to physiological states of cells, one requires the analysis of SSB at the level of individual cells by *in situ* nick translation (ISNT). In the principle of ISNT, the DNA strand with breaks is elongated in the presence of biotin-11-dUTP by *E. coli* DNA polymerase I at the nicked sites. The biotin moieties incorporated into the newly synthesized strand are visualized enzyme-immunohistochemically with horseradish peroxidase-labeled anti-biotin antibody. In this article, I will firstly describe the details of optimization of the ISNT reaction for its full implementation, using nicked λ phage DNA, which was fixed onto nitrocellulose filters, as well as using fresh frozen sections of rat testis and small intestine. Subsequently, I would like to demonstrate the presence of two types of SSB, which can be practically distinguished by protease-dependency of the staining; one is readily detected by ISNT without any deproteination steps (protease-independent type), and the other requires the protease treatment to be detected by ISNT (protease-dependent type). In our case, the single-strand breaks of DNA observed in terminally differentiated cells as well as the cells undergoing necrosis were protease-independent type. On the other hand, the DNA breaks in the cells undergoing replicative DNA synthesis and apoptosis were regarded as the latter type. Consequently, ISNT should be regarded as a useful molecular histochemical tool to categorize naturally occurring SSB.

Key words: **DNA single-strand breaks, *In situ* nick translation, Terminal differentiation, DNA synthesis, Cell death**

I. Introduction

DNA single-strand breaks (SSB) have been implicated to play important roles in the regulation of cell proliferation [36, 41], cell differentiation [2, 8-10] and cell death [6, 14, 15, 17, 18, 43]. Actually, we have previously found that ultraviolet light (UV) irradiation induces the differentiation of HL-60 cells to acquire NBT reducing activity [31]. Also, it has been found that human peripheral blood lymphocytes are transiently activated by UV-irradiation to express a set of genes including PCNA [31]. Since UV-irradiation induces SSB transiently when the pyrimidine dimers are excised and repaired [33], the results seemed to support the hypothesis that the occurrence of SSB is involved in the alteration of gene expression. For a better understanding of the biological significance of SSB, it seems essential to correlate the occurrence of SSB with the changes in physiological states of each cell. In spite of its apparent importance, however, only a few efforts have been made to analyze the occurrence of SSB at the level of individual cells.

In order to analyze the occurrence of SSB directly at a level of individual cells, *in situ* nick translation (ISNT) could be a powerful tool [8, 19, 29], one which was originally developed to investigate the region of actively transcribed chromatin [14, 22] and was performed with radioactive compounds. However, the radioactive ISNT is generally time-consuming and expensive, and it is hard to localize the sites of nicks precisely on the subnuclear basis. That is why, more recently, the application of nonradioactive ISNT has become common [7, 13, 18, 24, 35, 42]. Furthermore, the needs for such a technique have recently been increasing rapidly in the particular field dealing with apoptotic cell death, which is characterized by a special occurrence of DNA strand breaks in the early stage of cell death commitment [6, 37, 43]. Therefore, now it seems appropriate to review the recent findings in this

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methodology, as one aspect of molecular histochemical methods [27].

In this article, I will describe the details of the methodological aspects of nonradioactive ISNT, including the recommended protocol. Subsequently, I would like to demonstrate the presence of two types of SSB through a variety of examples, and finally to stress the potential usefulness of ISNT in the biomedical research field.

II. Principle of Nonradioactive ISNT

"Nick translation" is a very common method to label cDNA to produce molecular biological probes, in which cDNA is nicked by DNase I digestion and at the nicked sites a new DNA strand is synthesized using the intact strand as a template. Basically, ISNT is based on the same principle to that of "nick translation". However, DNase I digestion is not needed.

The sites of SSB in nuclei can be firstly recognized by *E. coli* DNA polymerase I, which has three enzymatic activities; 5′ to 3′ DNA polymerase activity, 3′ to 5′ exonuclease activity and 5′ to 3′ exonuclease activity. As shown in Fig. 1, using the activities, the polymerase usually adds a new complementary deoxyribonucleotide to an intact free terminal 3′-OH residue in the presence of dATP, dGTP, dCTP and TTP, depending upon the base sequence of the intact template strand. At the time of elongation, the newly synthesized DNA strand can be labeled with haptenic nucleotide analogues such as biotin-11-dUTP [8, 18, 29] and digoxigenin-11-dUTP [7] when one of the substrates, TTP is replaced with these analogues. Finally, the incorporated hapten molecules are reacted with horseradish peroxidase (HRP)-labeled anti-hapten antibody and visualized enzyme-immunohistochemically. In "nick translation", the polymerase reaction is usually conducted around 15°C to ensure the high fidelity of the synthesized base sequences to the template strand. In ISNT, however, such a high fidelity in base complementarity between a newly synthesized strand and the template strand is not required. Therefore, we could accomplish a remarkable enhancement of detection sensitivity of ISNT by conducting the reaction at 37°C, which allows us to generate the "snapback" synthesis of DNA.

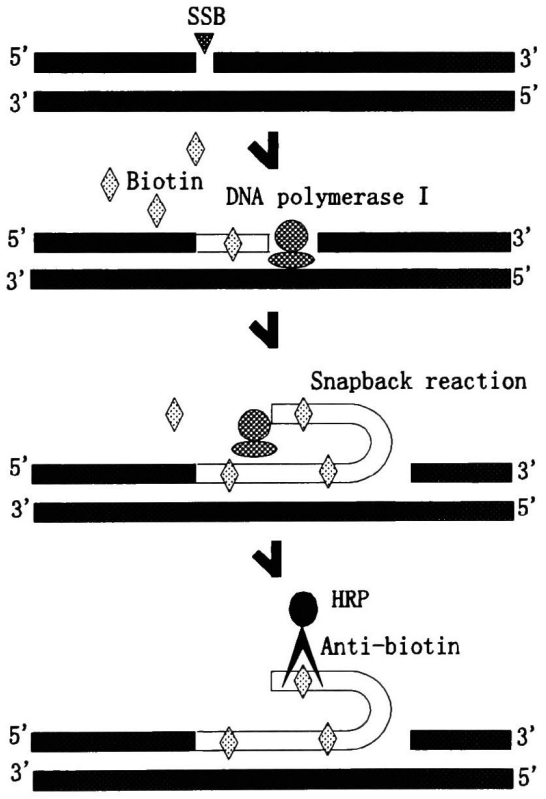

Fig. 1. Principle of nonradioactive ISNT. See the text for the detail. SSB: DNA single-strand break, biotin: biotin-11-dUTP.

III. Optimization of ISNT

In order to establish the optimal conditions for ISNT reaction, nitrocellulose filters spotted with λ phage DNA (10 ng/2 μl/spot) were prepared as described previously [25]. The λ phage DNA had been digested with DNase I at a concentration of 1–100 ng/ml, which was dissolved in a solution consisting of 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT) and 50 μg/ml BSA, for 15 min at 37°C. The digestion was terminated by the addition of 50 mM EDTA. After baking the filters at 80°C for 2 hr, they were reacted with various concentrations of *E. coli* DNA polymerase I (Toyobo or Takara, Japan) at 25°C or 37°C for various incubation periods in the nick translation buffer (NTB) containing 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM DTT, 50 μg/ml BSA, and each of 20 μM of dATP, dGTP, dCTP and biotin-11-dUTP (or TTP). After washings with 50 mM Tris/HCl buffer (pH 7.5), the filters were incubated with 5% BSA in PBS for 1 hr at room temperature (RT; 25–27°C). To detect the biotin molecules incorporated into DNA, the filters were reacted with HRP-labeled anti-

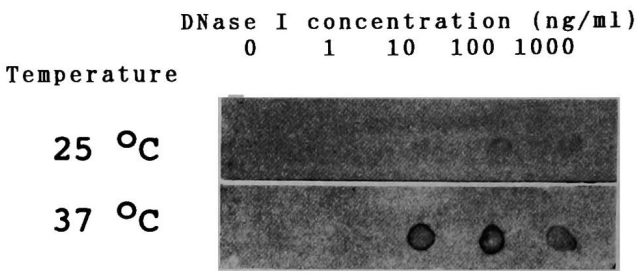

Fig. 2. Effect of temperature upon the detection sensitivity of SSB by the ISNT reaction on a nitrocellulose filter. Each 10 ng of λ DNA nicked with DNase I at various concentrations was spotted onto a nitrocellulose filter and reacted with DNA polymerase I at 200 U/ml for 3 hr at 25 or 37°C. Clearly, a 37°C-incubation gave a higher sensitivity.

Table 1. *Quantification of signals for SSB visualized by ISNT*

Dose (Gy)	*Arbitrary unit*	*P*
0	1.0±1.6	—
2.50	1.6±2.1	N.S.
5.00	4.3±2.1	$P<0.05$
10.00	6.2±2.9	$P<0.025$

Each 10 ng of λ DNA, which was irradiated with X-ray at various doses, was placed onto a nitrocellulose filter and subjected to the nick translation reaction. After visualization of SSB, staining densities of the spots were measured by an image-analyzer. Arbitrary units were expressed as mean±S.D. (n=4). For statistical analysis, the Student's *t*-test was used, compared to the non-irradiated group.

biotin antibody (Zymed or Vector Lab., USA) (1 : 100) diluted with 5% BSA in PBS, for 2–3 hr at RT. The sites of HRP were visualized by utilizing 3,3′-diaminobenzidine, H_2O_2, $CoCl_2$ and $NiSO_4(NH_4)_2SO_4$ [27].

As a result, the highest sensitivity was obtained with 200 U/ml DNA polymerase I, as compared to that with 50 U/ml and 100 U/ml of the enzyme. When the reaction time was varied from 1.5 to 15 hr, the staining intensity reached a maximum level in 3 hr-incubation. Moreover, the incubation at 37°C gave a more intense signal than that at 25°C, as shown in Fig. 2.

To evaluate the sensitivity of ISNT under optimal conditions, λ phage DNA (50 μg/ml) was irradiated with X-ray at 0.427 Gy/min for various intervals. It was known that 0.5 Gy induces 500 single-strand breaks per diploid cell nucleus [5]. The irradiated DNA was fixed onto nitrocellulose filters, and then the filters were subjected to ISNT, as described above. The stained filters were analyzed by an image analyzer (Model SP-500 (S): Olympus, Japan) for quantification of signal intensity. As shown in Table 1, the signal density of each spot was read by the image analyzer and expressed as an arbitrary unit. At doses higher than 0.5 Gy, the measured signal density became significant and increased linearly. Our tentative calculation revealed that the ISNT permits us to detect at least about 2.6×10^5 single-strand breaks/mm^2.

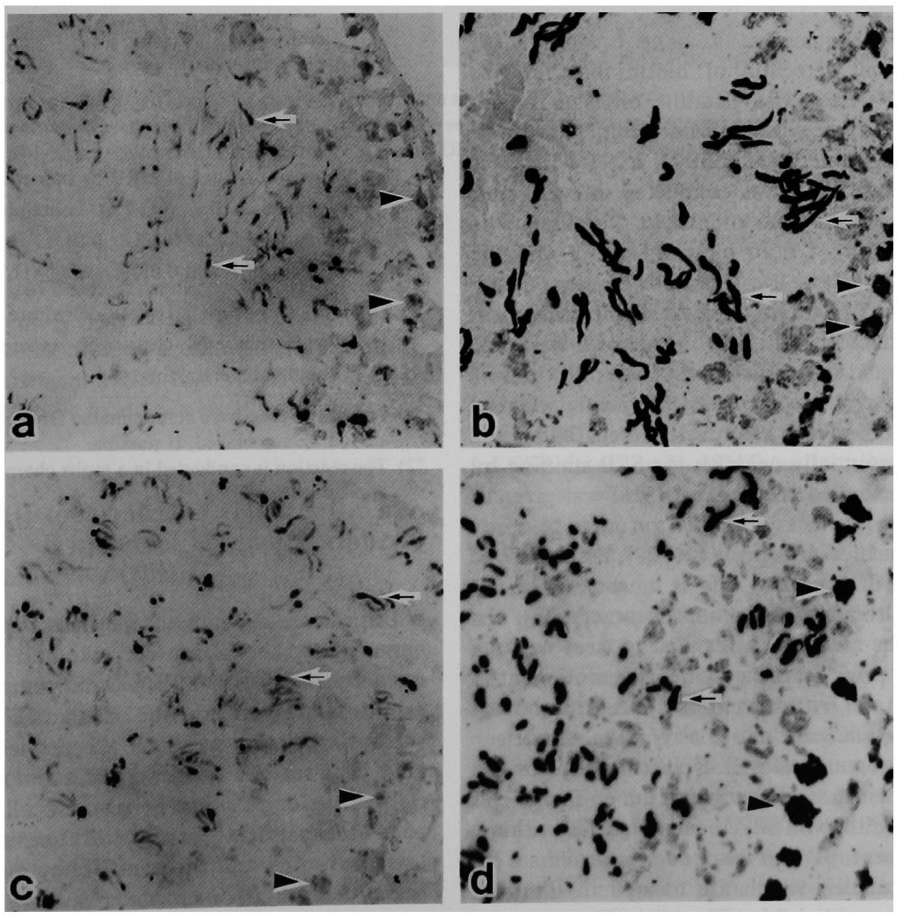

Fig. 3. Effects of fixatives and proteinase K digestion upon SSB signal in fresh frozen sections of rat testis. The frozen sections of rat testis were fixed with 4% PFA in PBS (**a**), acetone (**b**) or ethanol/acetic acid (**c** and **d**). ISNT was performed in the presence of biotin-11-dUTP, as described in the text. A section (**d**) was treated with proteinase K prior to the polymerase reaction, while the others (**a**, **b** and **c**) were not. Arrow head: spermatogonium, arrow: elongated spermatid. ×350.

IV. Application of Nonradioactive ISNT to Tissue Sections and the Presence of Two Types of SSB

For a full implementation of the ISNT reaction on tissue sections, various attempts to optimize the conditions for tissue processing including fixation and treatment with protease were carried out, using fresh frozen sections of rat testis. The use of testis sections seemed appropriate because the nuclei of maturing spermatids were known to contain SSB [19]. The tissues were obtained from Wistar male rats weighing 150–160 g, which were killed by cervical dislocation. The tissues were excised and were quickly frozen with OCT compound (Miles, USA) and stored at −80°C. The fresh frozen sections (5–6 μm) were cut and placed onto 3-aminopropyltriethoxysilane (Sigma, USA)-coated or gelatin-coated glass slides [27]. The fresh frozen sections were fixed in various fixatives such as acetone, 4% paraformaldehyde (PFA) in PBS and ethanol/acetic acid (3 : 1, v/v) at RT for 20 min. Then the sections were treated with or without proteinase K (Sigma) in PBS (1 μg/ml, 37°C, 15 min). After several washings with PBS, they were immersed in 50 mM Tris/HCl (pH 7.5). ISNT was conducted for 3 hr at 37°C in the NTB containing 200 U/ml of DNA polymerase I. The procedure for immunohistochemical detection of biotin moieties was described above. After visualization of signals, the sections were cleared, dehydrated and mounted without counterstaining. As a negative control, ISNT with TTP in place of biotin-11-dUTP was conducted in every run. Moreover, some sections were stained in the absence of DNA polymerase I or of both biotin-11-dUTP and the enzyme.

As for selection of fixative, the best signal/noise ratio was obtained by ethanol/acetic acid. As shown in Fig. 3, the nuclei of elongated spermatids in the frozen sections of testis were stained without protease treatment, whereas the nuclei of the other cells, e.g. Sertoli cells and spermatogonia, were essentially negative for SSB staining by ISNT. The recommended protocol for ISNT was given in Chart 1.

As well documented previously [26, 28], tissue nucleic acids are masked with proteins and it must be hard for DNA polymerase I to attain at the sites of target nicks. In order to unmask the nicks, we attempted to treat sections with proteinase K prior to the polymerase reaction. As shown in Fig. 3, in the frozen sections of testis, the treatment with proteinase K gave rise to a remarkable nuclear staining of spermatogonia as well as spermatids. In the fresh frozen sections of small intestine, the epithelial cell nuclei of tip parts in the villi were heavily labeled without the protease treatment. When treated with proteinase K, the epithelial cell nuclei, which are located in the proliferating zone, became visible (Fig. 4). These results indicate that there are two types of SSB; one is readily detected by ISNT (protease-independent type) and the other requires the protease treatment for the staining of SSB (protease-dependent type). In addition, the nuclei of nerve cells, skeletal muscle cells and chondrocytes were intensely stained for SSB without the protease treatment.

Iseki [19] reported the cellular distribution of SSB in various rat tissues by ISNT, where the signals in proliferative cells such as spermatogonia and intestinal epithelial cells in the crypt parts were specifically detected without protease treatment. The results seemed apparently contrasted to ours and the controversy may be explained by the difference between the fixatives; he used ethanol/acetone (1 : 1, v/v), instead of ethanol/acetic acid. Actually, it was known that when tissue sections were fixed with ethanol/acetone, the tissue DNA turned to be insensitive to DNase I digestion, indicating that the fixative may

Chart 1. *The recommended procedure for nonradioactive ISNT*

1. Cut fresh frozen sections (5–6 μm), place them onto silane-coated glass slides and air-dry.
2. Fix with ethanol/acetic acid (3 : 1) for 20 min, and wash 3 times with PBS*1 for 5 min each time.
3. If necessary, treat with proteinase K (1 μg/ml) at 37°C for 15 min, and wash 3 times with PBS for 5 min each time.
4. Immerse in 50 mM Tris/HCl buffer (pH 7.5) and keep until to use.
5. React with *E. coli* DNA polymerase I (200 U/ml) at 37°C for 3 hr in the nick translation buffer consisting of 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 50 μg/ml BSA, 20 μM dATP, 20 μM dGTP, 20 μM dCTP and 20 μM TTP or 20 μM biotin-11-dUTP*2.
6. Wash 3 times with 50 mM Tris/HCl buffer (pH 7.5) for 5 min each time.
7. Immerse in 0.3% hydrogen peroxide in methanol for 15 min.
8. Rinse once with PBS, and incubate with PBS containing 500 μg/ml normal goat IgG and 5% BSA for 1 hr*2.
9. React with HRP-goat anti-biotin antibody (1 : 100), diluted with 5% BSA in PBS for 2 hr*2.
10. Wash 4 times with 0.075% Brij 35 in PBS for 15 min each time.
11. Visualize the sites of HRP by immersing in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.5 mg/ml 3,3′-diaminobenzidine/4HCl, 0.01% hydrogen peroxide, 0.025% $CoCl_2$ and 0.02% $NiSO_4(NH_4)_2SO_4$ for 5–6 min.
12. Wash with deionized water, dehydrate with serial upgraded ethanol solutions, clear with xylene, and then mount without counterstaining.

*1) In order to inactivate contaminated DNase I activity, all solutions should be autoclaved, if possible.
*2) The reaction is conducted in a moist chamber humidified with an appropriate solution.

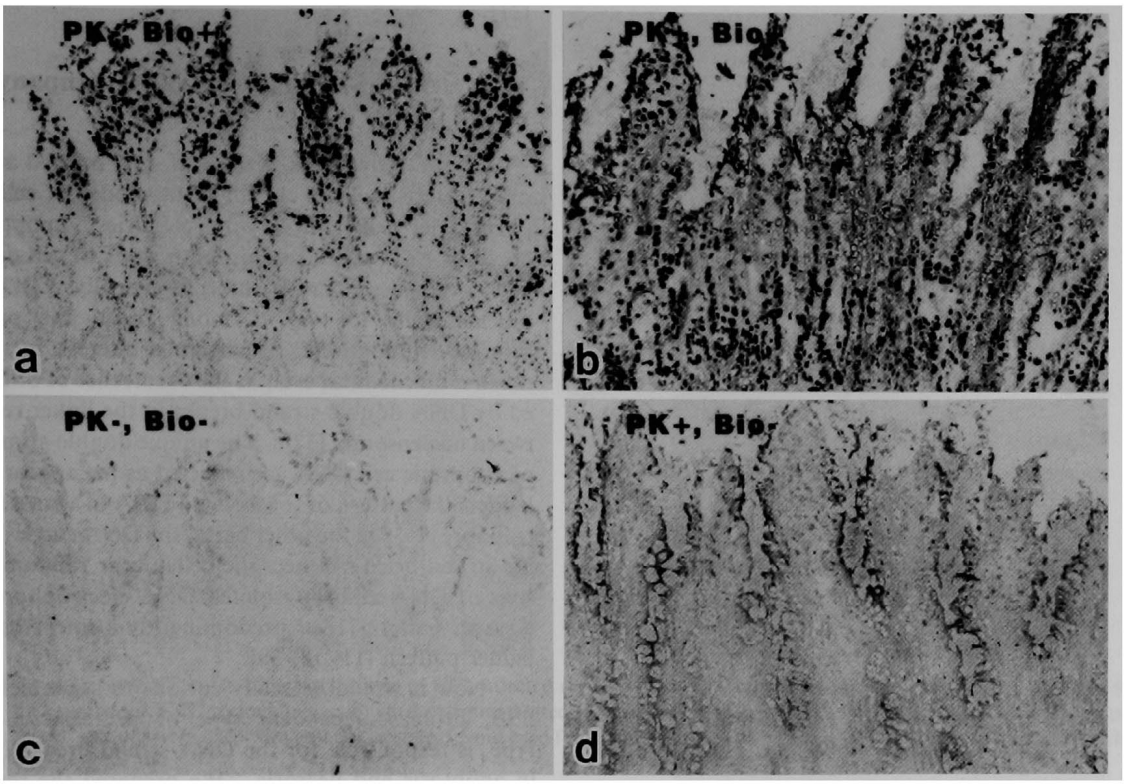

Fig. 4. Effect of proteinase K digestion upon SSB signal in fresh frozen sections of rat small intestine. The fresh frozen sections of rat small intestine were fixed with ethanol/acetic acid and used for ISNT. Some sections (**b** and **d**) were digested with proteinase K and the others (**a** and **c**) were not. As control, some sections (**c** and **d**) were reacted with DNA polymerase I in the presence of TTP in place of biotin-11-dUTP. $\times 100$.

cause drastic structural changes in chromatin [19]. In addition, Kishimoto *et al.* [24] reported that the combination of acidic fixative such as ethanol/acetic acid and following air-drying caused artificial DNA injury. However, our results revealed that acidic fixative in itself was not a cause of the DNA injury.

V. Molecular Histochemical Evidence for the Absence of SSB in Intact Human Peripheral Blood Lymphocytes (PBL)

As an example of application of ISNT, we addressed whether resting PBL have SSB endogenously or not, because the substantial discussion on the presence or absence of SSB in resting PBL was held. Human blood was drawn from healthy male volunteers and separated in Ficoll-Paque density-gradient centrifugation. The PBL fraction was collected and washed with PBS. Finally, the PBL were suspended in 0.25 M sucrose. To intend to induce SSB in the nuclei of PBL, an aliquot of cell suspension was irradiated with UV at a dose of 10 J/m^2, and then the cells were washed with MEM. The final pellet was resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and allowed to repair UV-induced DNA damage at 37°C under 5% CO_2 in air. At various time-points, an aliquot of cell suspension was centrifuged onto gelatin-coated glass slides by Cytospin (Shandon: Southern Products, USA), as detailed previously [30]. During the repair process of DNA damages induced by UV irradiation, SSB are supposed to occur transiently in human PBL. The Cytospin preparations were processed for SSB detection by ISNT, as described above.

As shown in Fig. 5, no detectable level of SSB was observed in the normal resting PBL, irrespective of the protease treatment. Two hr after UV irradiation, the signal for SSB appeared in the nuclei of PBL and the staining was not dependent upon proteinase K digestion, while the protease treatment resulted in an increase in the intensity of the staining. One day after UV irradiation, most of the staining for SSB disappeared.

As a matter of fact, there are many conflicting reports on the presence of SSB in resting human PBL [3, 4, 16, 20, 21, 38]. For the first time, Johnstone and Williams [20] reported the presence of SSB in resting PBL by means of the nucleoid sedimentation technique, and insisted that the SSB must be ligated prior to re-initiation of proliferation in response to lectins. Later, although the results were confirmed by Greer and Kaplan [16] and Prasad *et al.* [38], the other groups failed to demonstrate the presence of SSB by different methods [3, 21], strongly arguing that the SSB

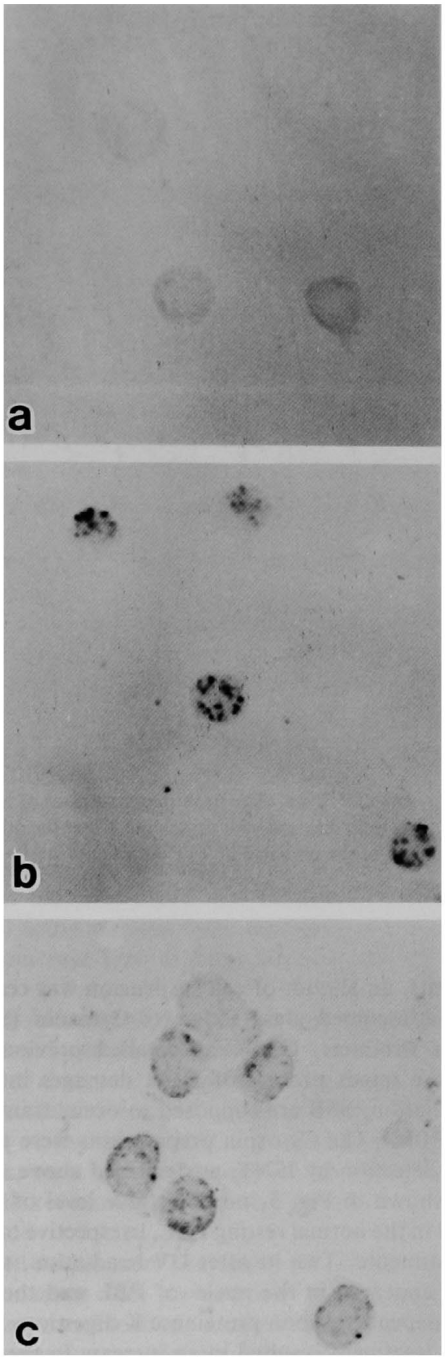

Fig. 5. *In situ* detection of SSB in human PBL irradiated with UV. Human PBL were irradiated with UV at a dose of 10 J/m². Two (**b**) or 24 hr (**c**) later, an aliquot of the cell suspension was cytocentrifuged onto the glass slides, treated with proteinase K and used for ISNT. Non-irradiated PBL were also used (**a**). ×880

are illusional and an artifactual product by the nucleoid sedimentation technique. As shown here, the histochemical approach with ISNT, never used for this subject, has clearly presented the absence of SSB in PBL, in consistent with the results of Boerrigter *et al.* [3] and Jostes *et al.* [21].

VI. Recent Findings on SSB Accompanying Cell Death

In the early 1970s, Kerr *et al.* [23] opened a new field on cell death. They classified the modes of cell death in eukaryotic cells into two categories; apoptosis and necrosis. The differences in both types of cell death have been well documented morphologically [23, 24] and biochemically [6, 43]. Among them, the pattern of genome degradation is of particular interest; in apoptosis, the first biochemical event is the occurrence of SSB, followed by DNA double-strand breaks in the linker regions between nucleosomes [37]. The unique double-strand breaks of apoptotic cell DNA are reflected as the appearance of a ladder-like pattern of the isolated DNA in agarose-gel electrophoresis. On the other hand, the DNA single- and double-strand breaks in necrotic cells occur randomly at any sites of DNA and the isolated DNA electrophoreses with a smear pattern [1] or predominantly a smear but a faint ladder pattern [11, 18, 39].

Now it would be really curious to see which type of SSB, protease-dependent type or protease-independent type, is responsible for the DNA strand breaks occurring in apoptosis and necrosis. We prepared the rat thymus with an intraperitoneal injection of hydrocortisone (10 mg/100 g BW, 2 hr) and the rat liver with an intraperitoneal injection of CCl_4 (100 μl/100 g BW, 28 hr) as a model of apoptosis and necrosis, respectively. Using these models, we found that necrotic SSB belong to protease-independent type, while apoptotic SSB are protease-dependent one [18], as shown in Fig. 6. Therefore, we should say that ISNT is a convenient molecular histochemical tool to discriminate both types of cell death without losing morphological details. Unfortunately, however, the effectiveness of this method was limited to the frozen sections, because no difference in nuclear staining of apoptotic or necrotic cells was observed in paraffin-embedded sections.

In addition, *in situ* TdT or TUNEL [12, 17, 32], in which terminal 3′-OH of DNA is added repeatedly with a nucleotide by terminal deoxynucleotidyl transferase, is frequently used to identify apoptotic nuclei as a method to detect the double-strand breaks of DNA preferentially. When *in situ* TdT was applied to the above models [18], essentially a similar staining tendency to that of ISNT was found, whereas the sensitivity was much less than that of ISNT. Surprisingly, a specific nuclear staining was also observed in the frozen sections of CCl_4 treated liver, where no hepatocytes with typical apoptotic features were detected by electron microscopy. These findings raise a warning that the detection of double-strand breaks as well as single-strand breaks may not exactly mean the occurrence of apoptosis. Especially in the case of paraffin embedded sections, we would be better to be more careful in interpretation of the staining results since there is, at

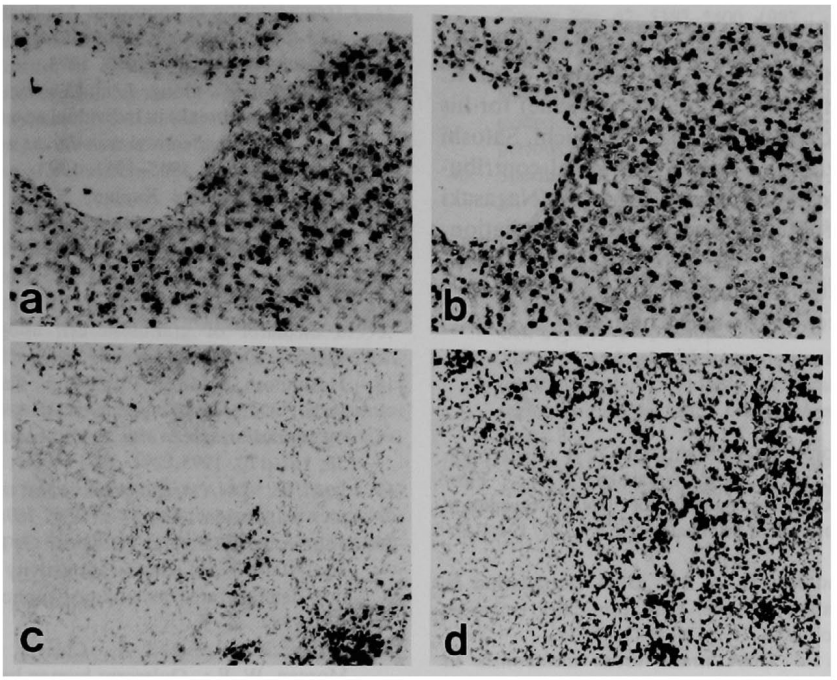

Fig. 6. SSB signals by ISNT in fresh frozen sections of CCl_4-injected rat liver (**a** and **b**) and of hydrocortisone-injected rat thymus (**c** and **d**) [18]. In this experiment, CCl_4-treated liver and hydrocortisone-treated thymus were used as a model for necrosis and apoptosis, respectively. The fresh frozen sections were fixed with ethanol/acetic acid and used for ISNT. Some (**b** and **d**) were treated with proteinase K and the others (**a** and **c**) were not. $\times 100$.

least in these particular cases, no difference in protease-dependency of signals of DNA strand breaks between apoptosis and necrosis. In addition, Gold *et al.* [13] reported that early stages of necrosis were preferentially detected by ISNT, whereas *in situ* TdT was slightly more sensitive for the detection of apoptosis.

VII. Conclusive Remarks

In the present article, I have demonstrated that there are at least two types of SSB, which are naturally occurring at various stages of the life cycle of cells. Particularly, it was of interest that the SSB in the cells undergoing replicative DNA synthesis belong to protease-dependent type, whereas terminally differentiated cells contain a significant number of SSB of protease-independent type. When ISNT was applied to detect dying cells, the single-strand breaks in the nuclei of cells undergoing apoptosis were categorized into protease-dependent type and those in the necrotic cell nuclei were into protease-independent type. Moreover, the breaks generated temporarily in the DNA repair process were categorized to the latter group.

The presence of SSB in terminally differentiated cells was repeatedly discussed, whereas the biological significance has not been clarified. In our case, we found SSB in spermatids, intestinal epithelial cells in the tip parts of villi, nerve cells and chondrocytes, which will never proliferate thereafter. Taken together with the data on lens cells and skeletal muscle cells into account, I am tempted to hypothesize that the signal detected protease-independently by ISNT in frozen sections is a possible marker of terminal differentiation, which is characterized by non-proliferative nature. However, other circumstantial evidence is also necessary to discriminate from necrotic SSB.

Compared to the SSB in terminally differentiated cells, the dependency of detectability of SSB in replicating cells upon protease treatment was remarkable. It does not mean that a number of SSB sites in replicating DNA is not sufficient to be detected by ISNT. Rather, it appears to indicate that proteolytic unmasking of the sites of SSB is required for accessibility of the SSB to DNA polymerase I. These results allow us to speculate that the single-strand breaks formed during replicative DNA synthesis are strictly protected for the following reaction such as ligation and reconstitution of nucleosomal structure, but those in terminally differentiated cells are not intended to be ligated in the near future and not protected.

ISNT was originally developed to identify the active chromatin region in tissue preparations, and currently has been recognized as a powerful and convenient histochemical tool to detect SSB in individual cell nuclei, particularly by combining use of nonradioactive analogue such as biotin-dUTP and digoxigenin-dUTP. Now, in this paper, I have offered the further utility of ISNT in discrimination of naturally occurring SSB.

VIII. Acknowledgment

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