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DNA INTERSTRAND CROSSLINKS IN NORMAL HUMAN SKIN VISUALIZED BY ELECTRON MICROSCOPY

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Abstract. Normal human skin was investigated for DNA interstrand crosslinks by a denaturation electron microscopic method. Skin biopsy materials from the thighs of 8 healthy volunteers were separated into epidermis and dermis. DNA from each layer was isolated, purified and studied in the electron microscope. The presence of DNA interstrand crosslinks in normal human skin was demonstrated. The number of crosslinked DNA molecules and the density of crosslinking within the molecules were determined. Crosslinks were found in 0.8 % of the scored molecules and were located almost exclusively in epidermal DNA. The intramolecular density of crosslinking showed a wide range of variation in each volunteer and between the volunteers.

Key words: Epidermal DNA; DNA interstrand crosslinks; EM visualization

Interstrand crosslinks are covalent linkages between complementary strands of the DNA double helix. Such crosslinks may occur after the exposure of DNA to various physical and chemical agents (4, 6, 7, 8, 10, 11). Detection of interstrand crosslinks has been carried out mostly by hydroxylapatite column chromatography (4), alkaline sucrose gradient centrifugation (6), CsCl gradient ultracentrifugation (8) and renaturation kinetics (11).

In the present study we have investigated the DNA from normal human skin regarding its content of interstrand crosslinks. To visualize the crosslinks we have used a denaturation electron microscopic method which, in contrast to the above-mentioned techniques, has the advantage of being very sensitive in detecting small amounts of DNA and revealing both the location and the density of crosslinking.

By utilizing this method we have demonstrated interstrand crosslinks in DNA isolated from normal human skin.

MATERIALS AND METHODS

Patients and sampling

Punch biopsies, 2 mm in diameter, were obtained from 8 volunteer subjects. 5 of these were healthy individuals and the remaining 3 had localized non-inflammatory skin conditions not affecting the thighs. The ages ranged between 22 and 69 years (Table 1). In each subject, biopsies were obtained from the thigh using ethyl chloride for local freezing anaesthesia and the skin specimens were immediately frozen and stored in liquid nitrogen.

Separation of dermis and epidermis

The frozen skin biopsies were placed in 0.25 ml buffer A and 1 mg trypsin (Trypure Novo, 225000 NF trypsin) for 45 min at 37°C (9). Buffer A contained: 0.5 M EDTA, 0.14 M NaCl, 0.003 M KCl, 0.008 M HNa_2PO_4, 0.001 M glucose, pH=8.6. The epidermis was gently removed from the dermis as one continuous layer with two tweezers and placed in 0.25 ml buffer A. The dermis was cut into 6–10 pieces with scissors and placed in 0.25 ml buffer A. Throughout the rest of the procedure the test tubes containing epidermis and dermis were run in parallel.

DNA isolation

Each sample was incubated for 10 hours at 37°C after addition of 10 μl collagenase (2000 U/ml, type III, Sigma). Cell lysis and deproteinization were done by addition of 50 μl 40% Sarkosyl (NL 97, recrystallized, Ciba-Geigy) and 8 μl pronase (10 μg/ml, Protease type III, Sigma) followed by incubation for 5 hours at 50°C. 270 μl 2 M $NaClO_4$ was added and the DNA was extracted with 1100 μl chloroform/octanol (9 : 1). It was precipitated by addition of 1100 μl 96% ethanol and resuspended in 0.25 ml SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH=7.0). 15 μl heat-treated ribonuclease (1 mg/ml, Ribonuclease A, type III, Sigma) was added and incubated for 30 min at 37°C followed by addition of 8 μl pronase (10 μg/ml, Protease, type III, Sigma) and incubation for 90 min at 37°C. The DNA was once again extracted with chloroform/octanol (9 : 1) and precipitated by ethanol.

Isolation of leukocytes from human blood

1 ml buffer A was added to 10 ml blood from 3 volunteer subjects and centrifuged at 5000 rpm for 10 min. The

Table I

Pat. no.	Sex/Age M=male F=female	Epidermis	Dermis	No. of non-crosslinked molecules	No. of crosslinked molecules	No. of crosslinked molecules/ No. of non-crosslinked molecules (%)	Intramolecular percentage of crosslinking
1	M/30	+		272	3	1.1	53.1, 23.5, 56.3
			+	212	2	1.0	50.7, 71.7
2	M/69	+		219	3	1.4	18.5, 11.0, 10.3
			+	156	0	0	
3	F/45	+		227	0	0	
			+	227	0	0	
4	F/35	+		402	8	1.9	13.3, 56.8, 46.3, 69.0, 71.4,
			Sample lost	–	–	–	36.3, 34.1, 67.8
5	F/22	+		238	1	0.5	7.9
			+	191	0	0	
6	M/26	+		190	5	2.6	21.9, 30.6, 27.9, 23.6, 36.7
			Sample lost	–	–	–	
7	M/22	+		418	5	1.2	73.9, 0.01, 76.1, 9.0, 2.5
			+	357	0	0	
8	M/30	+		421	1	0.2	3.1
			+	144	0	0	

intermediate layer, containing leukocytes, was isolated and placed in 0.25 ml buffer A. The DNA was isolated as described previously.

Electron microscopy

After removal of the ethanol, the precipitated DNA was resuspended in 100 μl of 99% formamide (Fluka, recrystallized according to (12)), containing 4 M urea, 10 μl of 30% glyoxal (Merck). This solution was heated for 1.5 min at 68°C thus ensuring total denaturation of the DNA (1). After cooling to room temp., 2 μl of buffer R (2 M Tris-HCl, 0.5 M EDTA and 2.7 mg/ml cytochrome *c* (type III, Sigma)) was added to the sample, and it was spread on a hypophase containing 150 ml double-distilled water in a 12×12 cm square Petri dish. Spreading conditions were essentially as described by Davis et al. (5). Grids (coated with 1% Formvar (Merck)) were stained in uranyl acetate and rotary shadowed at a 7° angle in an Edwards vacuum evaporator using a Pt/Pd 80:20 filament. They were examined in a Philips 201 electron microscope.

Upon total denaturation, partially crosslinked DNA molecules appear double-stranded at crosslinked regions and single-stranded at non-crosslinked regions (bubbles).

Measurements of molecules

This was done from the positive prints at a magnification of 34 900. Magnification was currently checked by use of calibration grids. A Hewlett-Packard 9825 A calculator and a 9864 A digitizer (Carlsberg Research Centre) were used. For measurements of double- and single-stranded length, the molecules had to be unambigous in their interpretation and to be free from breakages in their single-stranded or double-stranded regions. The smallest detectable single-stranded region (bubble) was about 150 basepairs in length.

RESULTS

Results obtained in the 8 investigated volunteers are shown in Table I. A total of 2 387 DNA molecules in epidermis and 1 287 DNA molecules in dermis were scored in the electron microscope. Molecules containing less than three crosslinks were not interpreted as being crosslinked, since cross-over points of DNA could form from random molecular display. We found 26 crosslinked molecules in epidermis and 2 crosslinked molecules in dermis. Fig. 1 shows a crosslinked molecule. Positive prints similar to Fig. 1 were used for measurement of intramolecular density of crosslinking.

Seven out of 8 subjects contained crosslinked molecules in epidermis, whereas only one out of 6 subjects had such molecules in dermis. The average percentage of DNA molecules containing (3 or more) crosslinks was 0.8 ± 0.8 (mean ± S.E.M.), range 2.6 to 0, $n=8$.

Crosslinked molecules were accurately measured for their apparent single-stranded and double-stranded lengths. The percentage of crosslinking

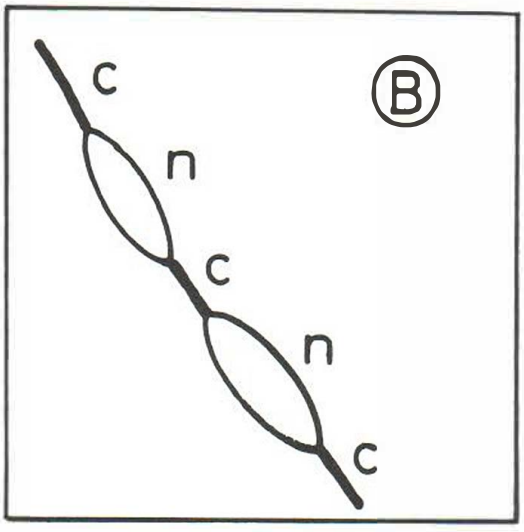

Fig. 1(A) shows a crosslinked DNA molecule from the epidermal skin layer of a healthy person. The intramolecular percentage of crosslinking in this molecule was 16.2%. The total magnification is 33 000×. The bar has a length of 1 kilobasepair equalling 0.3 μm. (*B*) is a schematic diagram of a crosslinked DNA molecule. *c* indicates crosslinked and *n* non-crosslinked regions of double-stranded DNA.

signifies the length of double-stranded DNA (crosslinked DNA) expressed as a percentage of total molecular length. This parameter is indicated in Table 1. The percentage of crosslinking is seen to vary extensively, within and between the volunteers.

For a control, DNA from human leukocytes was purified, denatured and spread for electron microscopy. 511 DNA molecules were scored, and none of them contained crosslinks.

DISCUSSION

By the use of a sensitive electron microscopic technique, we have visualized and evaluated the amount of DNA interstrand crosslinking in human skin. The crosslinks are found even in completely normal human skin, an observation not previously reported.

Crosslinks were found in both dermis and epidermis, though they were almost exclusively located to the latter. The outermost location and the high cell turnover rate of epidermis may account for this finding, by rendering epidermal DNA more susceptible than dermal to crosslink inducing factors. A wide range of variation in the intramolecular percentage of crosslinking (Table 1) occurred within different skin DNA molecules from each volunteer, and between the volunteers. This may be ascribed to the heterogeneity in size of the examined DNA molecules or to the high complexity in base composition of human DNA. Furthermore, variation due to individual factors, age, and sex may exist. However, the material was too small for us to evaluate these possibilities.

As a check on our procedure, DNA from human leukocytes was investigated in an identical way. No crosslinks were found in this material. Furthermore, we have previously been able to introduce and graduate DNA crosslinks in a study on purified *Tetrahymena pyriformis* bulk DNA exposed to 8-methoxypsoralen and UV-A light (1).

The biological consequences of DNA interstrand crosslinks are not understood. In vitro investiga-

tions have shown that such crosslinks inhibit nucleic acid (3) and protein synthesis (2).

However, the presence of interstrand DNA crosslinks in normal human skin is not inconsistent with these in vitro observations. The number of crosslinks that we found may be too small to interfere with nucleic acid and protein synthesis. Furthermore, the observed DNA defects may be reversed in vivo through the action of DNA repair mechanisms.

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