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〔原著〕 STUDIES ON DNA REPAIR IN CELLS FROM XERODERMA PIGMENTOSUM, BLOOM'S SYNDROME AND COCKAYNE SYNDROME PATIENTS

Takayuki KOJIMA*

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SUMMARY

To investigate DNA repair mechanisms defective in Bloom's syndrome (BS), Cockayne syndrome (CS) and xeroderma pigmentosum (XP) patients, including variant form (XPV), the mechanisms in fibroblasts from BS in siblings, three CS patients and five XP patients were compared with those from normal donors. Cloning efficiency method and host cell reactivation assay (HCR) were employed to examine the ultraviolet ray (UV) susceptibility. Excision repair was estimated by unscheduled DNA synthesis (UDS) using an autoradiographic method and repair replication synthesis using a density labeling and equilibrium centrifugation method. So-called post replication repair was estimated by an alkaline sucrose gradient method. XP showed depressed excision repair capacity. XPV was shown to be slightly sensitive in HCR and defective in post replication repair. Two cases of BS had no abnormalities revealed by the tests employed. One CS case showed depressed UDS and repair replication, although the clinical CS findings were typical. Results suggest the heterogeneity in DNA repair mechanisms in XP, BS and CS is more complex than is now known.

Key words: DNA repair, xeroderma pigmentosum, Bloom's syndrome, Cockayne syndrome

Abbreviation: XP: xeroderma pigmentosum, XPV: xeroderma pigmentosum variant, BS: Bloom's syndrome, CS: Cockayne syndrome, EMEM: Eagle's minimal essential medium, FCS: fetal calf serum, UV: ultraviolet ray, CF: colony formation, EDTA: ethylenediamine tetraacetic acid, PBS: phosphate-buffered saline, HCR: host cell reactivation, HSV-I: herpes simplex virus type I, UDS: unscheduled DNA synthesis, [^{3}H] dThd: tritium thymidine, BrdUrd: 5-bromodeoxyuridine, FrdUrd: 5-fluorodeoxyuridine

Introduction

There exist a number of recessively inherited photosensitive diseases which are frequently accompanied by malignant neoplasma, neurological and immunological abnormalities: Xeroderma pigmentosum (XP), Bloom's syndrome (BS), Cockayne syndrome (CS) and others.

XP is an autosomal recessive disorder showing extreme photosensitivity from an early age. Many

* Department of Dermatology, School of Medicine, Chiba University, Chiba 280.
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* 千葉大学医学部皮膚科学講座
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pigment freckles, telangiectases and hyperkeratoses occur on the sun exposed areas and eventually become various types of malignant neoplasms of the skin often causing death[1,2]. The eyes are also affected, showing conjunctivitis and often keratosis with corneal opacities[3]. Some XP patients display severe progressive neurological manifestation, which is called De Sanctis-Cacchione syndrome[4].

BS is an autosomal recessive inheritance, recognized by telangiectatic erythema of the face starting in infancy, sensitivity to sunlight, stunted growth, high frequency of both malignant neoplasma and sister chromatid exchanges and low level serum concentrations of IgA and IgM[5-10].

CS is also an autosomal recessive inheritance, showing sunlight sensitivity, dwarfism, mental retardation, micrccephaly, ataxia, retinal pigmentation, deafness, progeroid features and intracranial calcification[11,12].

It is believed that disturbance of the DNA repair mechanism may play some important common etiological role in all of these diseases[13,14]. In XP, it is proved that excision repair——one of the most important DNA repair mechanisms in human——is deficient[15]. DNA repair mechanisms in BS or CS, however, have not been investigated as much, and few findings have been reported concerning DNA repair abnormalities in these two syndromes. We therefore studied the DNA repair mechanisms on fibroblasts from BS and CS patients in comparison with those of XP.

Materials and Methods

Cell lines, virus and culture conditions

Cultures of fibroblast were derived from skin explants biopsied from unexposed parts of the body, principally from the inner parts of the upper arms of normal, XP, BS and CS subjects. The cell lines used were CR3CB, CR4CB and CR5CB from normal subjects. Their age and sex were 2/F, 20/F and 3/M, respectively. XP cell lines were XP1-KY derived from a 9yr old male and kindly provided by Dr. Hiraku Takebe and XP24TO derived from a 2yr old male and kindly provided by Drs. Misaki Kobayashi and Yoshiaki Satoh. XP4CB, XP5CB and XP6CB cells were taken from XP patients who visited our hospital: respectively a 49yr old male, a 59yr female and a 35yr male. BS cell lines, BS1CB and BS2CB were derived from siblings, 5 and 4yr old girls with Bloom's syndrome. Clinical features of these BS patients have been described in detail elsewhere[16]. CS cell lines, CS1CB, CS2CB and CS3CB were derived from siblings, 8 and 6yr old boys and a 7yr old girl with Cockayne syndrome. CS1CB and CS2CB were kindly provided by Dr. Katsuo Sugita.

The culture medium was Eagle's minimal essential medium (EMEM) containing 10% fetal calf serum (FCS) and antibiotics (60μg kanamycin/ml). Cell cultures were maintained in a light-free section of an incubator at 37°C under 5% CO_2 at high humidity. Cells were detached with 0.25% trypsin. The medium was usually changed every 3 day.

UV irradiation

For UV (ultraviolet ray) irradiation, three 15W Toshiba germicidal lamps were used in combination. An incident dose rate of 0.8 J/m^2/sec was determined just before each experiment, which was measured by an UV radiometer, UVR-254 (Tokyo Kogaku Kikai Co, Japan). Mock irradiation was done in the same way without UV illumination.

Cell survival measurements

Survival measurement in each fibroblast under UV irradiation was taken by colony formation (CF) assays. To study CF assays, each cell type was used after being subcultured at low cell density (about 1×10^4 cells/60-mm dish) to increase the plating efficiency of cell strains. The suspension of singly dispersed cells was prepared by treating monolayers with 0.125% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Cells were inoculated about 1000 to 2000 in 100-mm plastic dishes. The dishes were incubated for 20hr to allow the cells to attach, and then irradiated. After incubation for 2 weeks in fresh medium, plates were fixed with methanol and stained with Giemsa. Only colonies containing more than about 50 cells were counted.

The data were plotted as a "surviving fraction"

by dividing the number of colonies at each irradiation dosage by the number of colonies formed at mock irradiation. The D_0 value, which is the UV dosage required to reduce the CF activity from any point on the exponential portion of the surviving fraction curve to 37% of that point, was calculated from the slope of a long exponential portion in the curve.

Host cell reactivation assay

Host cell reactivation assay (HCR) was done basically according to Takebe et al.[17]. Logarithmicallygrowing cells were seeded into a 35-mm plastic dish and cultured until confluent state. One ml of virus [Herpes simplex virus type I (HSV-I)] suspension was irradiated with UV in a plastic dish. After adsorption of 0.5ml of virus suspension for 90min at 37°C under conditions of agitation every 30min, 2ml of fresh medium containing 0.25% human γ-globulin was added to the dish in order to prevent virus movement and further adsorption onto other cells through the medium. At the 3rd to 4th day, cells were fixed and stained and plaques were scored. The data were plotted as a "surviving fraction" of HSV-I by dividing the number of plaqus at each irradiation dosage by the number of plaques formed at mock irradiation.

Unscheduled DNA synthesis (UDS)

Autoradiographic measurements were employed. Cells (2×10^4) were inoculated into a chamber of the Lab-Tec Chamber Slide (4 chambers) and incubated until cells became confluent. To reduce the level of scheduled DNA synthesis, cells were incubated for 2hr in culture medium with 10mM hydroxyurea. Immediately after the UV irradiation ($20J/m^2$), cells were re-incubated in the pre-warmed medium containing tritium thymidine ([^{3}H] dThd) (10μCi/ml, 20Ci/mmole, New England Nuclear) for 3hr, washed with PBS 3 times and further incubated in the culture medium supplemented with 5μg/ml dThd for 1hr. Cells were then fixed in methanol and washed with 5% of cold trichloroacetic acid. Cell plates were dipped in Sakura NR-M2 autoradiographic emulsion, dried, and exposed for 10 days at 4°C. After development, cells were stained with Giemsa solution and grains on the nuclei of cells, excluding S-phase cells, were scored. The nucleus of S-phase cells was obscured by the very heavily accumulation of black grains in the emulsion over it.

DNA repair replication

The UV-induced DNA repair replication was measured essentially according to the procedure of Smith and Hanawalt[15], except where otherwise noted[19]. After incubation with medium containing 10μM of 5-bromodeoxyuridine (BrdUrd) and 1μM of 5-fluorodeoxyuridine (FrdUrd) for 1hr, cells that had been prelabeled with and without [^{32}P] phosphate (0.4μCi/ml, Japan Atomic Energy Research) were irradiated at $12J/m^2$ UV. Then cells were re-incubated with medium containing 10μM BrdUrd, 1μM FrdUrd, 2.5mM hydroxyurea and 10μCi/ml of [^{3}H]dThd. After being labeled for 3hr, cells were lysed by NET solution (0.01M NaCl, 0.01M EDTA and 0.01M Tris-HCl, pH 8.0) with 0.6% sodium dodecyl sulfate and 50μg proteinase K per ml for 4hr at 50°C. The cell lysates were extracted more than twice with 1.5 vol of chloroform : isoamyl alcohol (24 : 1), and the upper aqueous phase was dialyzed against NET solution. Each aqueous phase was made to a density of 1.7g/ml with CsCl and a final volume of 5ml, and then centrifuged in an SW 50.1 rotor of a Beckman L5-50 ultracentrifuge at 33000 rpm at 20°C for 62hr as described by Kappen and Goldberg[20]. After centrifugation the gradients were divided into 15 fractions, and the parental DNA peaks were detected by estimation of 260nm absorbance or by assaying ^{32}P radioactivity of each fraction. Three fractions of the parental DNA regions were combined and mixed with appropriate amounts of alkaline solution (1M K_2HPO_4 titrated to pH 12.5 with KOH) and CsCl (density, 1.7g/ml) and then recentrifuged under the same conditions as used for the neutral gradients. After the alkaline CsCl centrifugation, fractionation and detection of unreplicated parental DNA strands were done as above. Repair-replicated DNA was detected by assaying tritium radioactivity of each fraction as described by Smith and Hanawalt[18].

Alkaline sucrose gradient profils

The procedure was according to Gianelli et al.[21]. Cells (2.5×10^5) were inoculated in 60-mm plastic dishes and after 2 days, the dishes were washed with PBS and irradiated or mock-irradiated with UV (12.5 J/m^2). The treated cells were incubated in 5ml of warm medium for 1hr, and pulse-labeled with [^{3}H]dThd (33μCi/ml) for another 1hr, followed by washing with warm PBS and chasing for 2.5 hr with unlabeled medium containing thymidine and deoxycytidine (10μM each). In some experiments caffeine was added at 0.3mg/ml throughout the whole period after irradiation. After the chase, cells were scraped off the dishes with a piece of silicone rubber into 0.3ml of PBS containing EDTA (0.2%), centrifuged (600g for 5min at 18°C) and suspended in 0.5ml of EDTA solution. The suspension was then exposed to γ-irradiation (2000 rad) from a Co source. A solution of alkaline sucrose gradient (4.4ml, 5-20%) containing 0.1M NaOH and 0.1M NaCl was first overlaid with 0.2 ml of the cell suspension. Centrifugation was done for 75 min at 20°C and 40000 rpm in an SW 50.1 rotor of a Beckman L5-50 ultracentrifuge. After centrifugation, 10-drop fractions were collected from the bottom of the tubes and assayed for radioactivity in cold acid-insoluble material.

Results

UV sensitivity

XP1KY cells and XP24TO cells were consistently extremely sensitive to UV measured by CF ability. XP5CB cells XP6CB and cells were moderately sensitive to UV, while XP4CB cells were slightly sensitive. BS1CB and BS2CB cells showed normal sensitivity to UV, and CS1CB, CS2CB and CS3CB cells were intermediately sensitive (Fig. 1a, b). D_0 values in XP, BS, CS and normal cells were 0.3 J/m^2 of XP1KY, 0.4 J/m^2 of XP24TO, 2.9 J/m^2 of XP4CB, 2.7 J/m^2 of XP5CB, 2.3 J/m^2 of XP6CB, 4.1 J/m^2 of BS1CB, 4.4 J/m^2 of BS2CB, 1.2 J/m^2 of CS1CB, 1.4 J/m^2 of CS2CB and 1.3 J/m^2 of CS3CB, respectively. D_0 of normal cells were 4.0-5.0 J/m^2.

Host cell reactivation

Bacteria possess enzyme systems for repairing the damage caused by UV irradiation in the dark, in which damaged regions of DNA are excised and replaced. Damage to an infecting phage can also be repaired. Mammalian cells also have this capacity, known as HCR. HCR levels generally correlate with those of the capacity for repair replication[22].

Figure 2 shows the survival of UV-irradiated HSV-I in normal human skin fibroblast strains, in XP cell strains XP4CB, XP24TO and in BS strains BS1CB, BS2CB fibroblasts. XP24TO cells showed a lower HCR capacity than normal cells, as has been reported by several authers for other XP cells, although depression of XP4CB cells was mild. However, BS1CB and BS2CB cells showed normal HCR capability.

Unscheduled DNA synthesis

The first evidence for DNA repair in mammalian cells, using autoradiographic techniques, showed that UV irradiation induced the uptake of labeled thymidine into the DNA of mammalian cells that were not in the normal phase of DNA synthesis. This kind of DNA synthesis has been called UDS, and has been shown to be nonsemiconservative in nature. In XP, UV photoproducts are formed in DNA, but unlike those in normal cells, few are excised after irradiation. Thus, as a consequence of the reduced excision, only a few labeled thymidines are inserted into DNA by UDS in XP cells[23].

The capacity of UDS tested is presented in Fig. 3. The grains were scored least 100 nuclei of cells. Average numbers of grains per nucleus for normal control fibroblasts were 98 ± 28.0 (mean$\pm$SD). XP1KY and XP24TO cells, which are XP group A strains had extremely low capacity of UDS (<5% of normal). XP5CB and XP6CB cells had intermediate level of UDS (35% and 26% of normal, respectively), and the UDS capacity of XP4CB, clinically definite XP, was almost normal. BS1CB, BS2CB, CS1CB and CS2CB cells showed almost normal capacity of UDS (93-107%), while the level of CS3CB cells was depressed (81% of normal).

DNA repair replication

It has been reported that UDS and DNA repair

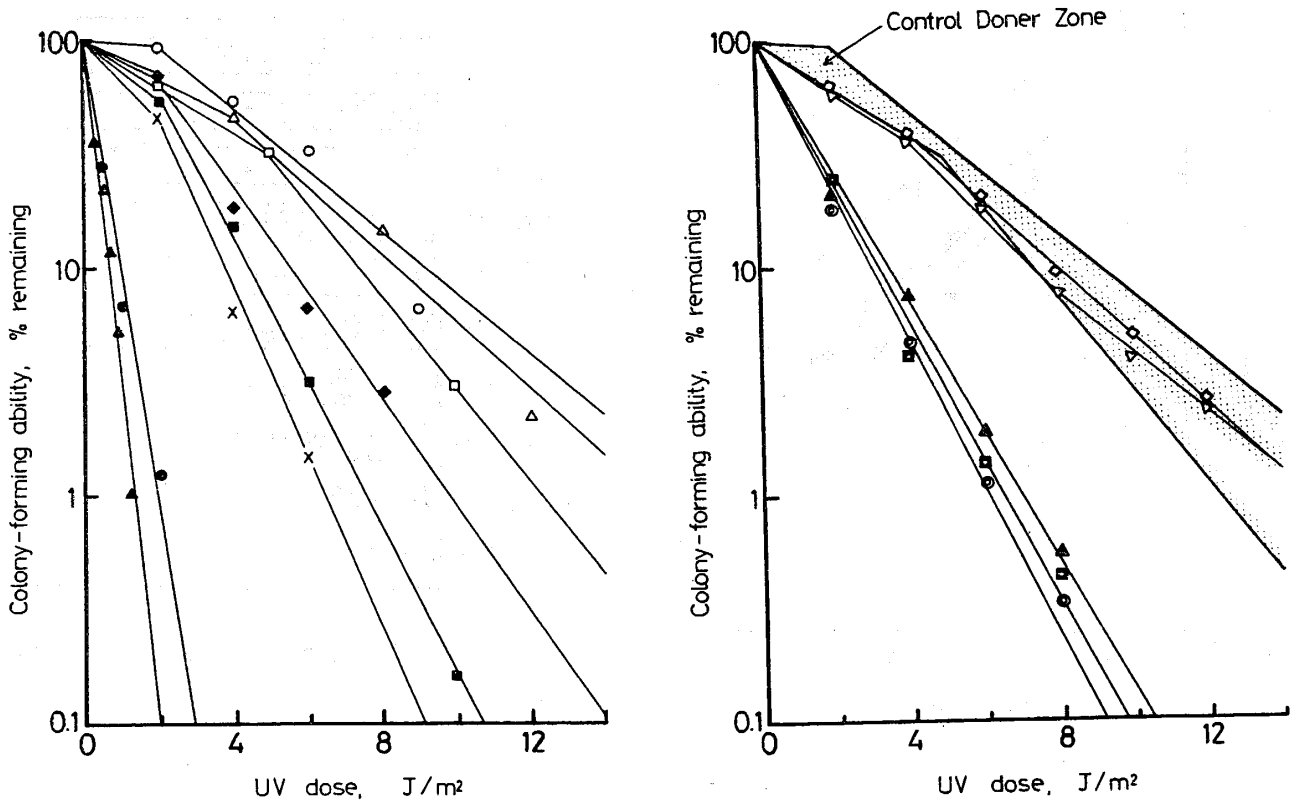

Fig. 1. a, b UV survival of normal (CR3CB, △ ; CR4CB, □ ; CR5CB, ○), XP (XP1KY, ▲ ; XP24TO, ● ; XP4CB, ◆ ; XP5CB, ■ ; XP6CB, ×), BS (BS1CB, ▽ ; BS2CB, ◇) and CS (CS1CB, ◎ ; CS2CB, ◬ ; CS 3CB, ▣) fibroblasts. 2-3 replicates were made for each dose level of irradiation. Each point shows the mean of 3 independent experiments. Cloning efficiencies of controls ranged from 10 to 30% for normal cells, from 3 to 10% for XP cells, from 3 to 15% for BS cells and from 10 to 15% for CS cells. The lower cloning efficiencies of some cell strains may reflect the fact that it was available for testing only at a late passage number.

replication were always detected together and that the extent of one roughly correlated with the other. The repair replication assay by recentrifugation of isolated parental density DNA can achieve clear separation of repair replication from semi-conservative replication. Therefore, to further confirm the level of repair activity of XP cells, BS cells and CS cells, we compared the extent of the replication in UV-irradiated XP cells with that in other irradiated cells. Increased peaks of UV-induced ^{3}H radioactivity coinciding with the peaks of the 260-nm absorbance and ^{32}P radioactivity of the unreplicated parental DNA was assayed as described in materials and methods. Repair replication capacity was estimated by $^{3}H/^{32}P$ radioactivity ratios of these fractions. The capacity of repair replication of XP1KY and XP24TO cells was extremely low (<5% of normal), but that of BS1CB, BS2CB and CS1CB cells was the same as that of normal cells (93%, 94%, 90% of normal, respectively). CS3CB cells showed a depressed level of repair replication activity (65% of normal) (Fig. 4).

Alkaline sucrose gradient

Lehmann et al. reported that there was one class of XP patients——XP variant——whose cells had completely normal excision repair process, while the cells had an abnormality in the manner of DNA synthesis after UV irradiation. In order to convert initially low-molecular-weight DNA synthesized in UV irradiated cells into high-molecular-weight DNA similar in size to that in untreated cells, these variant had much longer time than had normal cells. Furthermore, this slow conversion was drastically inhibited by caffeine, which had no effect

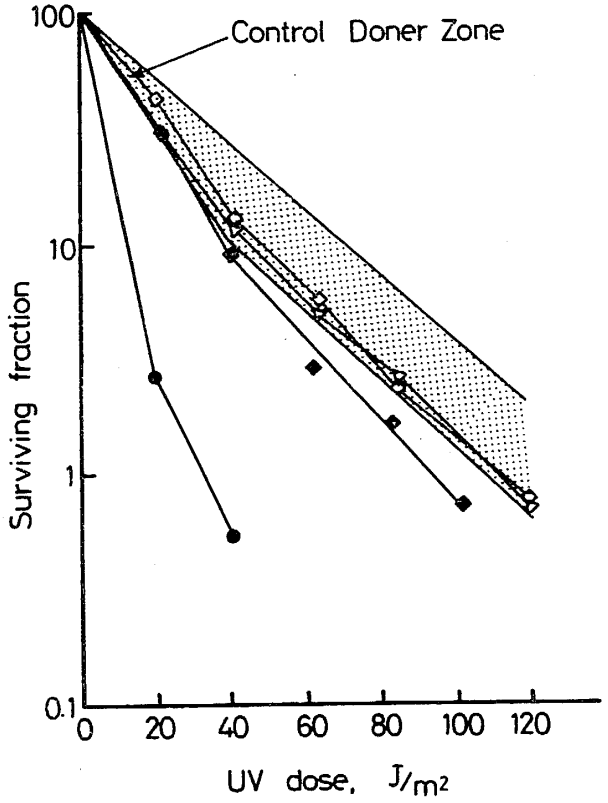

Fig. 2. Survival of UV-irradiated HSV in normal, BS (BS1CB, ▽; BS2CB, ◇) and XP (XP4CB, ◆; XP24TO, ●) cells. Control dishes without irradiation had 100 to 150 plaques per 35-mm dish. Two replicates were made for each dose level of irradiation and the same virus concentration. Each point represents mean results from at least 2 independent experiments.

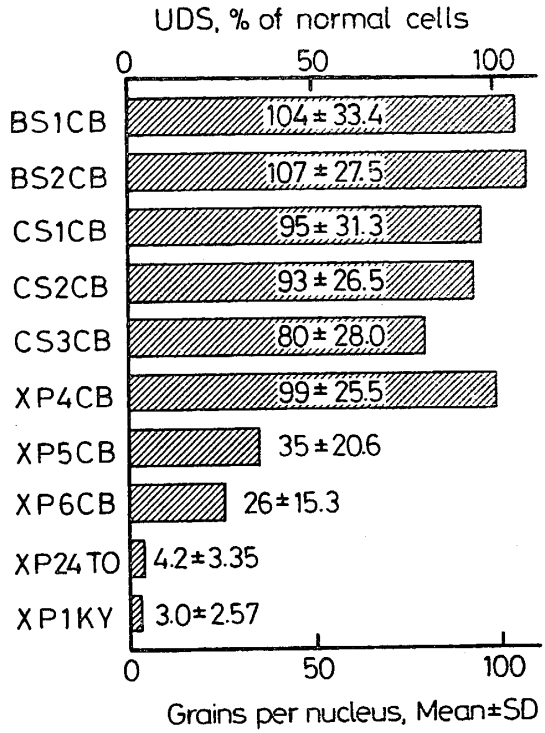

Fig. 3. UDS after UV irradiation at 20 J/m². The grains were scored at least 100 nuclei of cells. Average numbers of grain per nucleus for normal control fibroblasts were 98±28.0 (Mean±SD).

in normal cells[24]. So, we also performed this study for some cell strains. Fig. 5 showed the results of pulse and pulse-chase experiments with cells from normal fibroblasts, XP4CB, BS1CB and BS2CB cells. Closed circles showed the profiles of pulse-labeled DNA newly synthesized after 12.5 J/m² of UV irradiation. The profiles from normal and BS cell lines were very similarly situated in the center of the gradient. After a 2.5hr chase, all the DNA was near the bottom of the gradient (open circles) and this process was not changed by addition of caffeine (open triangles). While UV-irradiated XP4CB showed a quite different pattern. Namely, newly synthesized DNA by XP4CB after UV irradiation was considerably less than by normal cells and this inhibition was greatly enhanced by caffeine.

Discussion

Certain genetical photosensitive diseases tend to be accompanied by a tendency for malignancy, neurological and immunological abnormalities. XP is one of the most recognized and extensively investigated of these diseases. The etiology of XP is considered to be the result of disturbance to the DNA repair mechanism[15]. UV damage to cellular DNA results in the creation of thimine dimers[25], but this type of damage can be recovered by excision repair[26,27]. In XP patients, this excision repair mechanism is disturbed. XP is subdivided into groups A to I and variant type according to the excision repair capacity as measured by UDS[28,29]. It has already been established that XP cells have defects in their excision repair mechanism. Some authors have reported that variant types are deficient in post replication repair[24], while others have found them lacking in excision repair[30,31]. Our results showed that the XP cell lines——XP1KY,

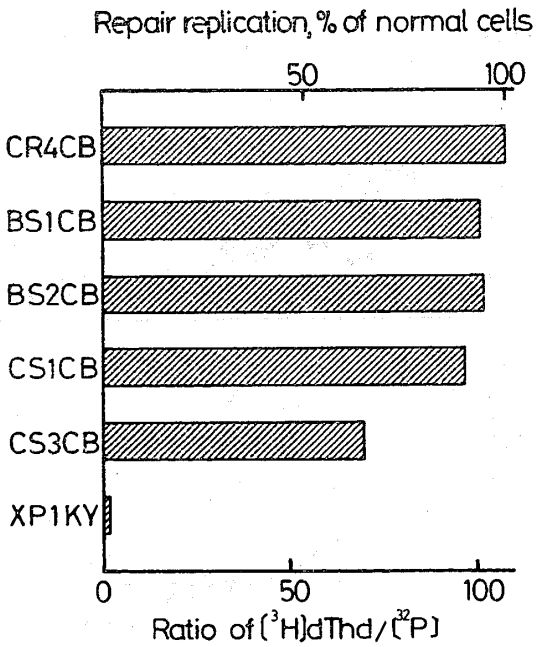

Fig. 4. Increased peaks of UV-induced ^{3}H-radioactivity coinciding with the peaks of ^{32}P radioactivity of the unreplicated parental DNA was assayed. Repair replication capacity was estimated by $^{3}H/^{32}P$ radioactivity ratios of these fractions. Result shows the mean of 2 independent experiments.

XP-24TO, XP5CB and XP6CB have an excision repair defect, as measured by UDS, HCR or repair replication assay. XP 4CB, which showed typical XP clinical features, had much the same capability of UDS as did normal cells, but it was slightly sensitive to UV, as determined by colony forming ability and HCR. Pulse-chase study by alkaline sucrose gradient centrifugation showed that newly synthesized DNA by XP4CB after UV irradiation was considerably reduced from that in normal cells and that this inhibition was even more enhanced by caffeine. Therefore, XP4CB cells have defects in the process of excision repair, as do other unknown DNA repair mechanisms.

The defects of DNA repair in BS have also been suggested[32-34]. Gianelli et al. reported that some BS cell strains had abnormalities in the process of alkaline sucrose gradient[21], while other reports stated that BS cell strain was not abnormal in UV sensitivity and also had normal levels of UDS[35,36]. However, the same strain of BS cells has never been investigated as extensively as in this report, and we attempted to clarify response patterns of CF, UDS, HCR, repair replication and alkaline sucrose gradient in UV-irradiated BS1CB and BS2CB cells. Normal levels were obtained in all of assays in both cell strains. Clinically, BS patients have abnormal sun-sensitivity, thus DNA repair in this disease may be defective in other unknown DNA repair systems.

In one of three investigated CS cases, we found low levels of UDS and repair replication, although the case had typical clinical features of CS. Therefore, there exists a close relationship of defects in the DNA repair mechanisms between XP and CS, although the phenotypes in these two genetic diseases are different.

Results suggest the heterogeneity in DNA repair mechanisms in XP, BS and CS is more complex than is now known.

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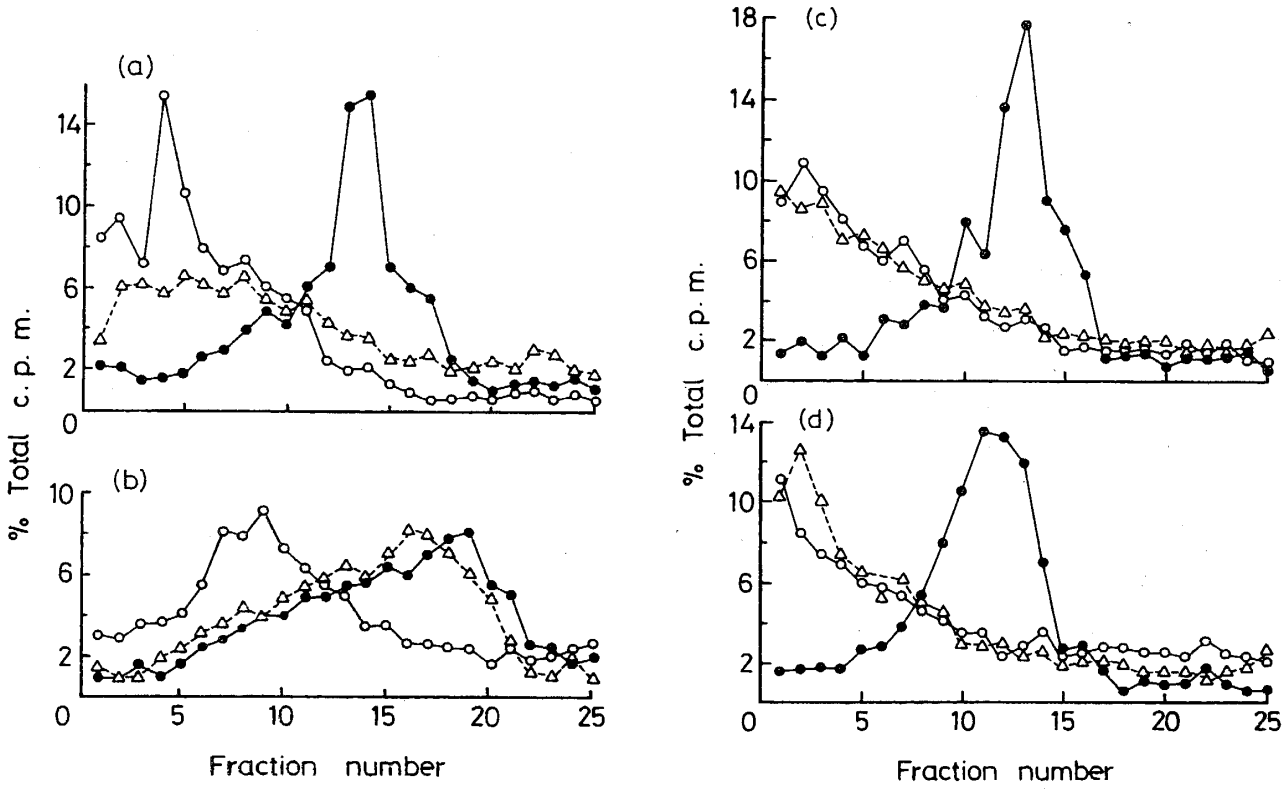

Fig. 5. Alkaline sucrose gradient profiles of normal fibroblasts (CR3CB) (a), XP4CB (b), BS1CB (c) and BS2CB (d) cells. After 12.5 J/m² of UV irradiation cells were pulse-labeled for 1hr with [³H]dThd and then chased for 2.5 hr with unlabeled dThd. Sedimentation was towards the left. Profiles similar to those shown in Fig. 5 were obtained in 2 independent experiments. (●): pulse-label, (○): chase, (△): chase with 0.3 mg/ml caffeine. Total cpm ($\times 10^{-3}$): (a) 5.2; (b) 5.9; (c) 6.9; (d) 5.4.

要　旨

高頻度発癌あるいは神経・免疫系の異常を伴う遺伝性光線過敏性皮膚疾患には色素性乾皮症（XP），Bloom 症候群（BS），Cockayne 症候群（CS）などが知られている。この中で XP はその病因として DNA 修復機構の1つである除去修復能の欠損していることが証明されているが，この周辺疾患ともいえる BS, CS の病因については不明な点が多い。そこでこれらの病因を解明する目的で，今日知られている DNA 修復機構の測定法を用いて以下の実験を行なった。対象には XP 5例，BS 2例，CS 3例の患者皮膚より培養した線維芽細胞を用いた。方法：1）コロニー形成法によって，紫外線感受性を測定した。2）ヘルペスウイルス（HSV-I）を用いた宿主細胞回復能（HCR）をみることによって紫外線感受性と除去修復能を測定した。3）不定期 DNA 合成（UDS），平衡密度勾配法による修復複製の測定によって除去修復能を検討した。4）アルカリ庶糖密度勾配法によって複製後修復能を検討した。結果：XP では紫外線高感受性があり，除去修復能の欠損を認めた。XP4CB は UDS が100%であり XP バリアントと考えられるが，除去修復能，複製後修復能いずれにも異常を認めた。BS では DNA 修復異常を示唆する報告もあるが，自験例ではどの測定法でも異常を認めなかった。CS3CB は典型的な CS の臨床症状を呈しながら除去修復能の低下を認めた。以上より，XP に相補性群があり多様性があるように BS, CS についても疾患の遺伝的多様性があり，従来知られている以上の DNA 修復機構の多様性があることが示唆された。

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