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Original Article

Brain vascular changes in Cockayne syndrome

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Cockayne syndrome (CS) and xeroderma pigmentosum (XP) are caused by deficient nucleotide excision repair. CS is characterized by cachectic dwarfism, mental disability, microcephaly and progeria features. Neuropathological examination of CS patients reveals dysmyelination and basal ganglia calcification. In addition, arteriosclerosis in the brain and subdural hemorrhage have been reported in a few CS cases. Herein, we performed elastica van Gieson (EVG) staining and immunohistochemistry for collagen type IV, CD34 and aquaporin 4 to evaluate the brain vessels in autopsy cases of CS, XP group A (XP-A) and controls. Small arteries without arteriosclerosis in the subarachnoid space had increased in CS cases but not in either XP-A cases or controls. In addition, string vessels (twisted capillaries) in the cerebral white matter and increased density of CD34-immunoreactive vessels were observed in CS cases. Immunohistochemistry findings for aquaporin 4 indicated no pathological changes in either CS or XP-A cases. Hence, the increased subarachnoid artery space may have caused subdural hemorrhage. Since such vascular changes were not observed in XP-A cases, the increased density of vessels in CS cases was not caused by brain atrophy. Hence, brain vascular changes may be involved in neurological disturbances in CS.

Key words: brain vessels, CD34, Cockayne syndrome, immunohistochemistry, xeroderma pigmentosum.

INTRODUCTION

Cockayne syndrome (CS) is a rare genetic disorder caused by deficient nucleotide excision repair (NER), and it is characterized by cachectic dwarfism, mental disability, microcephaly, cerebellar ataxia, retinal pigmentation, and neural deafness.[1] Neuropathological findings of CS patients show small cerebrum, tigroid leukoencephalopathy (dysmyelination), basal ganglia calcification, cerebellar atrophy and demyelinating peripheral neuropathy.[2] Many features of CS resemble those of premature aging, and hence, CS is considered as a progeroid syndrome.[3] Nancy and Berry divided 140 published cases into three types: type I, the most prevalent classical childhood disorder; type II, an infrequent, severe congenital, or infantile variant of the disorder; type III, atypical late onset of the disorder with prolonged survival.[4]

Xeroderma pigmentosum (XP) is an inherited neurocutaneous disorder caused by defects in the NER system.[1] Complementation studies using cell hybridization assays revealed the existence of eight genes in XP (groups A–G and a variant) and two in CS (A and B). NER includes global genome repair and transcription-coupled repair (TCR), which involves several XP genes (especially *XP-A* to *XP-G*) and two CS genes (*CSA* and *CSB*). In XP, the initial presentations are skin symptoms and progressive neurological manifestations, including cognitive and motor deterioration, neuronal deafness, peripheral neuropathy and brain atrophy, mainly in XP-A, XP-B, XP-D and XP-G cases.[2] The molecular basis of CS includes recessive mutations in *CSA* (*CKN1* or *ERCC8*) and *CSB* (*CKN2* or *ERCC6*) genes, but it has not been systematically mapped to the clinical phenotypes. We investigated neurodegeneration in autopsy cases of CS and XP-A.[2]

Arteriosclerosis in the brain[5] and cerebral vascular disorders have been reported in a few cases.[6] In order to characterize the brain vascular changes in CS, we compared the immunohistochemical changes in the brain vessels between CS cases and XP-A cases as disease controls.

MATERIALS AND METHODS

Subjects

The clinical study comprised five cases of clinically and genetically confirmed CS, six cases of clinically and

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Table 1 Summary of brain vascular changes in subjects

Subject	Age (years)	Sex	Cause of death	Brain weight (g)	Increase of subarachnoid small arteries	Twisted capillaries in the white matter	Density of CD34-immunoreactive vessels	
							Frontal Mean (SD)	Temporal Mean (SD)
Controls								
1	9	Male	Acute leukemia	N/A	(–)	(–)	29 (2)	21 (5)
2	13	Male	Chronic hepatitis	n/A	(–)	(–)	31 (1)	N/A
3	16	Male	Pneumonia	1505	(–)	(–)	29 (3)	25 (1)
4	20	Male	Malignant hyperthermia	N/A	(–)	(–)	26 (2)	25 (5)
5	29	Female	Guilain-Barre syndrome	N/A	(–)	(–)	38 (3)	33 (6)
6	36	Female	Thrombotic thrombocytopenic purpura	1475	(–)	(–)	37 (1)	30 (4)
7	47	Male	Acute leukemia	1400	(–)	(–)	35 (5)	38 (3)
8	55	Male	Lung cancer	N/A	(–)	(–)	38 (3)	36 (4)
9	60	Female	Breast cancer	1080	(–)	(–)	41 (7)	43 (4)
10	71	Male	Lung cancer	N/A	(–)	(–)	49 (3)	41 (4)
Cockayne syndrome								
1	7	Female	Pneumonia	295	1+	1+	44 (4)	41 (2)
2	15	Male	Renal failure	340	1+	1+	45 (4)	43 (4)
3	16	Female	Asthma	615	1+	(–)	48 (6)	N/A
4	18	Male	Renal failure	400	1+	1+	53 (5)	49 (5)
5	18	Male	Renal failure	414	1+	1+	44 (5)	45 (4)
Xeroderma pigmentosum group A								
1	19	Male	Candidiasis	580	(–)	(–)	28 (7)	26 (3)
2	19	Male	Renal failure	610	(–)	(–)	29 (11)	27 (2)
3	21	Male	Pneumonia	720	(–)	(–)	28 (3)	28 (2)
4	23	Female	Pneumonia	580	(–)	(–)	32 (2)	30 (3)
5	24	Female	Pneumonia	500	(–)	(–)	39 (4)	38 (4)
6	26	Female	Pneumonia	530	(–)	(–)	39 (3)	38 (4)

N/A, not assessed.

genetically confirmed XP-A, and 10 controls without any pathological changes in the CNS, aged 9–71 years (Table 1). The clinical and pathological findings in the CS cases 1–4 and XP-A cases 1–2 and 4–6 were reported previously.[7] The study was approved by the ethical committee of Tokyo Metropolitan Institute of Medical Science; informed consent was obtained from the patients' families before performing post mortem analyses.

Histochemistry and immunohistochemistry

The brains were fixed in buffered formalin solution; coronal sections of each formalin-fixed brain sample were cut and embedded in paraffin. Serial 6-μm-thick sections were cut from selected brain regions, including the superior frontal cortex, middle temporal cortex, basal ganglia and thalamus. HE staining and elastica van Gieson (EVG) staining were performed. After microwave antigen retrieval, each section was treated with CD34 (Nichirei, Tokyo, Japan) and rabbit polyclonal antibody to aquaporin 4 (AQP4; Santa Cruz Biotech, Santa Cruz, CA, USA). Each section was pretreated with proteinase K and mouse monoclonal antibody to collagen type IV (Col4: Sigma-Aldrich, St Louis, MO, USA). The antibody concentrations used for analysis were as follows: 1:1 (CD34), 1:100 (AQP4) and 1:500 (Col4). Antibody binding was visualized by means of the avidin–biotin immunoperoxidase complex method (Nichirei, Tokyo, Japan) according to the manufacturer's protocol.

Quantitative evaluation and data analysis

To determine the densities of the immunoreactive vessels, including arteries, veins and capillaries in the superior frontal cortex and middle temporal cortex, we determined the numbers of vessels immunoreactive for CD34 in five nonoverlapping microscopic subfields at 200-fold magnification by using a counting box (0.5 mm^2). Data were presented as mean (SD) and analyzed by nonparametric Mann–Whitney *U*-test to compare the results in different subjects. The level of significance was set at $P < 0.05$ to adjust for comparisons.

RESULTS

The results of HE and EVG staining and immunohistochemistry suggested that only CS patients had increased small arteries and arterioles in the subarachnoid space filled with fibrotic tissue and without arteriosclerosis (Table 1 and Fig. 1A). Further, small twisted and longitudinally running capillaries in cerebral white matter were observed in 4/5 CS patients but were not found in the brain

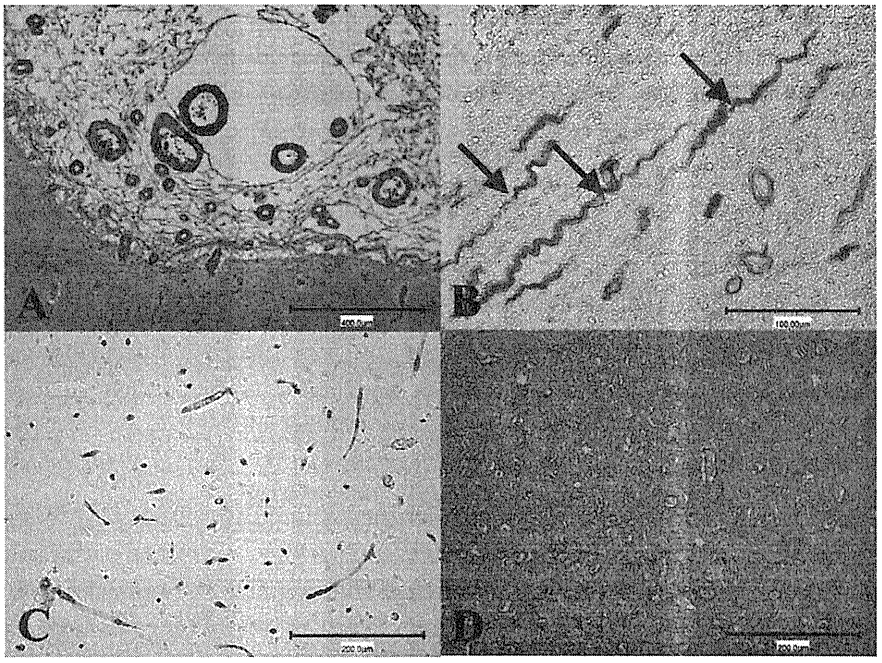

Fig. 1 Vascular changes in the brain of Cockayne syndrome (CS) patients. (A) Small arteries had increased subarachnoid space in CS case 1, elastica van Gieson staining. Bar = 400 μm. (B) Small twisted capillaries (arrows) were identified by immunostaining for collagen type IV in the cerebral white matter in CS case 4. Bar = 100 μm. (C) Vessels immunoreactive for CD34 in the middle temporal cortex in CS case 2. Bar = 200 μm. (D) Astrocyte processes were diffusely visualized by immunohistochemistry for aquaporin 4 in the cerebral white matter in CS case 4. Bar = 200 μm.

in either XP-A patients or controls (Table 1 and Fig. 1B). Twisted capillaries had more than five undulations, and were differentiated from functioning capillaries. In controls, the density of CD34-immunoreactive vessels in the frontal and temporal cortex of aged subjects was one-and-half times higher than that in teenagers, suggesting that the increase was age-dependent (Table 1). The density of CD34-immunoreactive vessels in CS cases aged less than 20 years was over 40 (Fig. 1C), which was equal to that in aged controls. The mean (SD) was 32 (4) in controls aged from 9 to 36 years, 47 (3) in CS cases, and 33 (5) in XP-A cases in the superior frontal cortex, respectively. The mean (SD) was 27 (4) in controls aged from 9 to 36 years, 45 (3) in CS cases, and 31 (5) in XP-A cases in the middle temporal cortex, respectively. The density of CD34-immunoreactive vessels in CS cases was significantly higher than those in controls and XP-A patients ($P < 0.05$). Furthermore, in CS patients, the number of capillaries increased around the calcified foci in the basal ganglia (data not shown). The vessel wall calcification was not found in the cerebral cortex, white matter or subarachnoid space. AQP4 immunostaining visualized the astrocyte processes around the vessels in all subjects (Fig. 1D) and there were no pathological changes in either CS or XP-A patients.

DISCUSSION

Cockayne syndrome and XP-A patients had severe brain atrophy (Table 1) and fibrosis in the enlarged subarachnoid space. However, the number of arteries and arterioles increased only in CS patients, suggesting excessive branching in the cortical arteries in CS. Similarly, in the subarachnoid space, increased density of CD34-immunoreactive vessels in the frontal and temporal cortex was observed in CS but not in XP-A patients. The reduction of brain area due to brain atrophy possibly leads to the apparent increase in the density of vessels. Nevertheless, since there was no increase in density in XP-A patients, the increased density of vessels in CS patients was not caused by the brain atrophy. Subdural hemorrhage is usually caused by tearing of the bridging cortical veins after head trauma, but traumatic aneurysms in the cortical arteries are a rare cause of such hemorrhages.[8] The increased number of subarachnoid and/or intracortical arteries may be a risk factor for subdural hemorrhage, which has been reported in CS patients.[6] A recent study showed that *CSB* mutant cells did not exhibit a normal reaction to hypoxia; these cells did not activate hypoxia-inducible factor-1 on the promoter gene due to which downstream events such as transcription factor IIB (TFIIB) recruitment did not occur.[9] Insufficient hypoxic response may disturb the induction of growth factors such as VEGF, suggesting the possible involvement of angiogenesis in CS. The analysis of brain vessels in fetal autopsy cases of CS might reveal the disturbances of angiogenesis during brain development.

Rapin *et al.* reported twisted microvessels consistent with so-called string vessels in the brains of adult CS patients.[5] We observed a similar morphological change in CS patients in our analysis. The absence of twisted microvessels in XP-A patients is noteworthy, and deficient NER is unlikely to have direct relationships with the

vascular changes in CS patients. Brooks *et al.* stressed that in addition to brain vascular lesions, there is an overlap in neurological symptoms, such as dysmyelination and brain calcification between CS and Aicardi–Goutières syndrome. They proposed that the vascular changes probably occur due to alterations in gene expression and may play a role in the generation of neurological abnormalities in both the diseases.[10]

CS is considered to be a progeroid condition since many symptoms of CS resemble premature aging. It is intriguing that arteriosclerosis was absent in the brain vessels in our CS patients, although this change has been pointed out in CS cases reported in the literature. In good accordance of our findings, the absence of atherosclerotic changes in the systemic arteries was reported in a 40-year-old patient with CS of probable type III.[11] Furthermore, neither senile plaques nor vascular beta-amyloid depositions were identified in the temporal lobe in three patients with CS of probably type I or II, two aged 2 and one aged 6 years, respectively.[12] In Werner syndrome (WS) associated with supposed accelerated aging, patients rarely show age-associated neuropathology and lack amyloid deposition, indicating the absence of extension of WS-associated aging in the CNS.[13] Although the increased occurrence of arteriosclerosis in the heart, aorta and kidney is a definite characteristic of CS and WS, further analysis of many autopsy cases is required to verify the facilitation of brain arteriosclerosis in both the disorders.

Mouse models for CS-A and CS-B show a TCR defect and increased photosensitivity in the skin. However, growth failure and neurological abnormalities are not predominant.[14,15] $Csb^{m/m}/Xpa^{-/-}$ double mutant animals show post natal growth retardation, ataxia, abnormal locomotor activity, progressive weight loss and early death.[16] However, in these model animals, brain vascular changes have not been examined in detail. Complete inactivation of NER by deletion of *XP-A* gene in animals does not cause CS-like neurodevelopmental and progeroid features, and it has been proposed that some of the CS features may be the outcome of defects in the transcription function of transcription/repair factor TFIIH and/or defective repair of oxidative DNA lesions.[17] We have investigated the involvement of oxidative stress in the brains of XP-A and CS autopsy cases.[2] Lipid peroxidation and protein glycation markers were found in the perivascular calcification areas in the globus pallidus and cerebellum more predominantly in CS than in XP-A patients. We found a similar deposition of oxidative stress markers in the calcification areas in the brain vessel walls in cases of pseudohypoparathyroidism and Fahr disease.[18] Since increased oxidative stress is known to cause vascular calcifications in bone and kidney diseases,[19,20] it is possible that oxidative stress may be involved in the generation of brain vascular changes in CS.

Our findings suggest that vascular changes in the brain may be involved in neurological disturbances in CS. A detailed investigation of the brain vessels may help us clarify the pathogenesis of neurological abnormalities.

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Letter to the Editor

Xeroderma pigmentosum complementation group G patient with a novel homozygous missense mutation and no neurological abnormalities

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Abstract: We describe an unusual xeroderma pigmentosum (XP) patient with a mutation in XP complementation group G, representing only the third reported Japanese XP-G patient. A 40-year-old men (XP3HM), born from consanguineous parents experienced sun sensitivity and pigmentary changes of sun-exposed skin since childhood. He developed a squamous cell carcinoma on his lower lip at the age of 40. He has neither neurological abnormalities nor Cockayne syndrome. The primary fibroblasts of the patient were hypersensitive to killing by UV (D_0 = 0.6 J/m^2) and the post-UV unscheduled DNA synthesis was 8% of normal. Host cell reactivation complementation analysis implicated XP complementation group G. We identified a novel homozygous mutation (c.194T>C) in a conserved portion of the *XPG(ERCC5)* gene, resulting in a predicted amino acid change; p.L65P. We confirmed that this genetic change reduced DNA repair thus linking this mutation to increased skin cancer.

Key words: DNA repair – mutation – skin cancer – ultraviolet – xeroderma pigmentosum group G

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Background

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by severe photosensitivity, abnormal pigmentation and a more than 10 000-fold increase in the frequency of cancers of sun-exposed skin and eyes (1–3). There are seven genetically different complementation groups, XP-A through XP-G, with defective nucleotide excision repair (NER) and XP variant with deficient translesion DNA synthesis. In Japan, 55% of XP patients belong to XP-A, a severe form with marked neurological degeneration. In contrast, in the US and Europe, 40% of XP cases are XP-C, in which patients only have cutaneous and ocular symptoms (4,5). There have been only 14 XP-G cases reported, including two Japanese XP-G cases (6,7). Most of the XP-G patients also have clinical features of Cockayne syndrome (XP/CS complex) or XP neurologic symptoms as well as cutaneous XP lesions (8,9), while two Japanese patients only had cutaneous lesions (7,10).

Questions addressed

Here, we report clinical and laboratory features of a new Japanese XP-G patient with a novel *XPG* mutation with no features of XP/CS complex or XP neurological disease.

Experimental design

Post-UV DNA repair and molecular studies using fibroblasts from the skin biopsy specimen of this patient were performed as described (11). Cultured fibroblasts XP3HM (this case), XP2OS(XP-A), XP20BE (XP-G) and N-3 (3-year-old normal donor) (6,11) were used.

Results

Case report

A 40-year-old men (XP3HM), with consanguineous parents, had marked freckling and telangiectasia on his face, upper chest and the dorsal aspects of both hands and the recent appearance of squamous cell carcinoma in his lower lip (Fig. 1). There was sparing of the buttocks and axilla. He had lifelong sun sensitivity but he never had a blistering sunburn. Pigmentary changes appeared at the age of 10. His physical and intellectual development were normal and neither neurological nor ocular abnormalities had developed. He had no difficulty in hearing. His minimal erythema dose was reduced (20 mJ/cm^2) (normal; 50–120 mJ/cm^2) (12).

Cell studies

The sensitivity to killing by UV of XP3HM cells (D_0 = 0.6 J/m^2) (11) was much greater than that of normal N-3 cells (D_0 = 5 J/m^2) but not as great as XP-A cells (Fig. 2a). The level of UV-induced unscheduled DNA synthesis (UDS) in XP3HM cells was only 8% of normal (11). Host cell reactivation assay (11) indicates that XP3HM cells are in XP complementation group G (Fig. 2b). Genomic DNA sequencing (13) revealed a novel homozygous T>C change in exon 2 of the *XPG* gene (c.194T>C, GenBank reference sequence X69978.1), resulting in a predicted amino acid change (p.L65P) (Fig. 2c). We constructed an expression vector (pXPGT194C) to determine whether this homozygous missense mutation is the cause of reduced DNA repair. After the transfection of pcDNA3-XPGT194C into XP20BE cells (harbouring different XPG mutations) (6), the DNA repair capacity was not restored to the normal level, while the expression of the wild-type *XPG* cDNA increased the DNA repair capacity of the XP20BE cells (Fig. 2d).

Conclusions

XP-G was first reported in 1979 (14). Of the 15 reported XP-G patients, including the present case, seven had XP/CS complex, two had XP neurological symptoms and six had only cutaneous XP symptoms (6,15). Skin cancers were reported in three of these six, all of whom were middle-aged Japanese patients, and in one

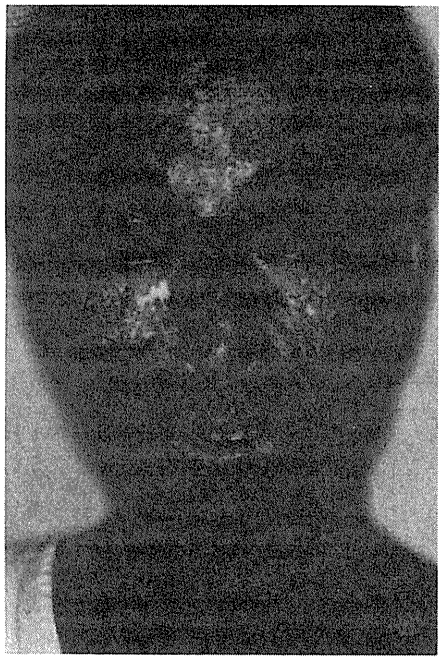

Figure 1. Clinical features of the face of the present case; XP3HM. Forty-year-old patient had marked hyperpigmentation and hypopigmentation of face and sun-exposed portion of the shoulders with some sparing upper neck. He also had marked cheilitis and atrophy of lips; however, he could open his mouth fully. A squamous cell carcinoma was removed from his lower lip.

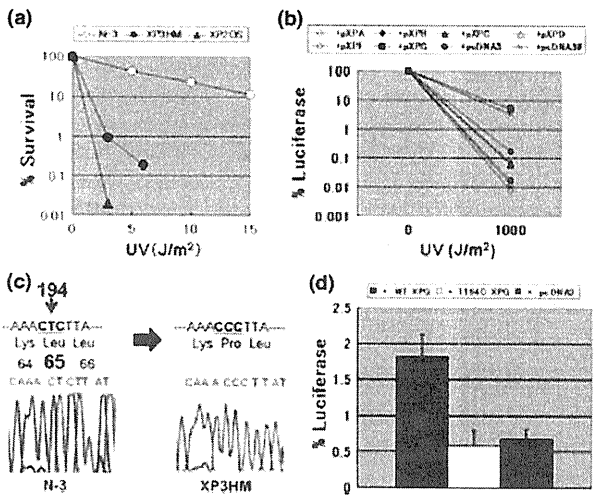

Figure 2. Laboratory analysis of XP3HM cells. (a) Post-UV survival of the cells: The D_0 value (a UV dose that results in 37% cell survival) of XP3HM cells (0.6 J/m^2) was much lower than that of the normal N-3 cells (D_0 value; 5.0 J/m^2). However, these cells were less sensitive than XP2OS (XP-A) (D_0 value; 0.3 J/m^2). (b) Host cell reactivation assay for the assignment of XP complementation group G in XP3HM cells: The cells were cotransfected with a UV-damaged *luciferase* gene expression vector along with expression vectors harbouring cloned wild-type XP cDNA. Increased luciferase activity was observed when the wild-type *XPG* cDNA expression plasmid was transfected into the cells from the patient, while luciferase activity was still very low after the transfection of expression vectors harbouring other *XP* cDNA (wt *XPA*, *XPB*, *XPC*, *XPD*, *XPF*) or the empty vector (pcDNA3). The restored DNA repair capacity after transfecting the wild-type *XPG* cDNA into the XP3HM cells reached the level observed in normal cells (x) after transfecting the empty vector (pcDNA3), (c) Nucleotide sequence analysis of the *XPG* gene in XP3HM cells: We identified a homozygous T to C change in exon 2 of the *XPG* cDNA (c.194T>C) with predicted amino acid change (p.L65P). (d) Host cell reactivation assay for the analysis of the DNA repair function of the c.T194C mutation in exon 2 of the *XPG* gene. We constructed a pXPGT194C plasmid using a pXPG plasmid and QuikChange Site-Directed Mutagenesis kit (Stratagene, CA, USA). After the transfection of pXPGT194C into the XP20BE cells, the luciferase activity (or the DNA repair capacity; DRC) was not significantly different from the control empty vector plasmid (pcDNA3), while the wild-type *XPG* cDNA increased the DRC of the patient's cells.

Western XP/CS complex case, XPCS4RO (6). All three Japanese XP-G patients (XP3HM, XP52HM and XP31KO) had similar clinical phenotypes of mild cutaneous features compared with those seen in XP-A or XP-C patients (7,10). They had their first skin cancers (squamous cell carcinoma of the lip, melanoma on the shoulder and basal cell carcinoma on the face), at the ages of 40, 54 and 32, respectively. This is older than many XP patients (1) but younger than the average age of skin cancer incidences in the Japanese general population (16–19). In addition, these three XP-G patients had no neurological symptoms or any evidence of CS. In contrast, seven of the 12 non-Japanese patients had clinical features of XP/CS complex and two had severe or a late-onset XP neurological phenotype (6).

The levels of post-UV UDS in the other Japanese XP-G patients (XP52HM and XP31KO) were 50% and 25% of normal, which was substantially higher than that observed in severe cases of XP-G or in the present case (8%). The mild XP symptoms in these three Japanese XP-G patients, therefore, may not be explained by the residual NER capacity, estimated by the level of UDS. The diversity of the phenotypes of XP-G may be due to different ethnic groups or to the different types and sites of mutations in the *XPG* gene.

The human *XPG (ERCC5)* gene contains 15 exons (13,20,21). The XPG protein functions as a nuclease in the NER, making incisions 3′ to the lesion releasing a 25–27 nucleotide DNA fragment containing the photoproduct (3). Mutations resulting in markedly truncated, inactive XPG proteins are found in XP/CS complex patients, while individuals with XPG without neurological disease have been found to have missense mutations that retain some functional activity (6).

The homozygous c.T194C change in the *XPG* gene in the present case is predicted to result in an amino acid change; p.L65P. This missense mutation is located in a PIN domain that is highly conserved in eukaryotes (Fig. S1) and can interact with both the XPB and XPD proteins (22). We confirmed that this mutation was related to decreased NER in the XP3HM cells using post-UV HCR analysis employing mutant XPG cDNA expression vector (Fig. 2d). This could explain the sun sensitivity and skin cancers in patient XP3HM. It is possible that p.L65P mutation preserves another function of XPG protein, such as transcriptional activity (22,23), thus preventing neurological degeneration. Genetic analysis has not yet been performed in XP31KO and XP52HM cells to determine their causative mutations. Additional studies will be needed to clarify the molecular basis of the mild clinical features of XPG cutaneous disease in the Japanese XP-G cases.

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Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Amino acid alignment of PIN domain of xeroderma pigmentosum complementation group G nuclease a structure-specific, divalent metal ion-dependent 5′ nuclease and homologues.

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ORIGINAL ARTICLE

Prenatal diagnosis of xeroderma pigmentosum group A in Japan

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ABSTRACT

We performed a prenatal diagnosis for 10 fetuses from nine unrelated Japanese xeroderma pigmentosum complementation group A (XP-A) families. All parents had at least one XP-A child (proband) with a homozygous founder mutation (IVS3-1G>C) in the *XPA* gene. A genetic analysis was performed by a restriction enzyme; *AlwNI* fragment length polymorphism of polymerase chain reaction (PCR)-amplified DNA, mostly from amniotic fluid (AF) and cultured cells established from AF. However, for the first family, we tried amniocentesis as well as chorionic villus sampling (CVS). Among the 10 cases, we confirmed the results of PCR-based genetic diagnosis by post-ultraviolet survival of amniotic cells in eight cases. Unfortunately, 6 weeks after CVS and 4 days after the amniocentesis in the first case we examined, the fetus died *in utero*, the reason for which remains unexplained. We prenatally determined two XP-A cases, six XP-A carriers and two wild-type fetuses, which appears to be consistent with Mendel's law.

Key words: amniocentesis, chorionic villi sampling, genetic analysis, prenatal diagnosis, xeroderma.

INTRODUCTION

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by severe photosensitivity, abnormal pigmentation and a more than 1000-fold increase in frequency of skin cancers in areas exposed to sunlight compared to the normal population.[1,2] The estimated frequency of the disorder is $1/10^6$ and $1/4–10 \times 10^4$ newborns in Western countries and Japan, respectively. Cultured cells from patients are very sensitive to killing by ultraviolet light (UV). There are seven genetically different complementation groups, XP-A through XP-G, with defective nucleotide excision repair (NER), and one NER proficient form with deficient translesion DNA synthesis (XP variant).[3] XP group A, a severe form of XP with marked neurological degeneration in addition to cutaneous features (XP neurological disease), is more common in Japan (~55% of Japanese XP-A patients) than in the USA and Europe, where 40% of XP cases are XP group C, a cutaneous type of XP without neurological abnormalities (XP cutaneous disease).[4]

The human gene responsible for XP group A, isolated in Japan, is 25 kb long and contains six exons.[5] Interestingly, 78% and 19% of the Japanese XP-A patients are homozygous or heterozygous, respectively, for a G>C transversion at the 3′ splice acceptor site in intron three (IVS3-1G>C) of the *XPA* gene, which causes abnormal splicing.[6,7] Conveniently, this mutation creates a new cleavage site for a restriction enzyme, *AlwNI*. This so-called "founder mutation" can be easily and rapidly detected by restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) amplified DNA fragments of the *XPA* gene after the digestion by *AlwNI* using various types of patient cells, such as white blood cells, buccal mucosal cells or cultured dermal cells. The second most common mutation found in Japanese XP-A cases is a nonsense mutation of exon six, which alters the 228th Arg codon to a stop codon (R228X). This mutation also creates a new restriction site, in this case for *HphI*, and can also be easily detected by PCR-RFLP. Approximately, 9% and 2% of the Japanese patients are heterozygous and homozygous, respectively, for this mutation and the *HphI* site in exon six of the *XPA* gene. Japanese patients with this mutation have less severe clinical disease than patients with the more common *XPA* mutation of the *AlwNI* site.

Therefore, in Japan, it is possible to diagnose most cases of the most common type of XP, XP-A, easily, rapidly and precisely by PCR-RFLP using these restriction enzymes. In addition, this diagnostic procedure is very useful not only for the diagnosis of patients in the clinic but can also be applied for prenatal diagnosis using amniotic fluid (AF) or chorionic villi as a source of prenatal DNA and the detection of XP-A carriers in people related to XP-A family.[8–10]

We have been carrying out prenatal diagnosis for pregnant mothers with one or more children with XP-A. In order to eliminate the possible misdiagnosis resulting from possible maternal contamination at the time of obtaining specimens, we have established a

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cell line from AF and studied the *XPA* gene by PCR-RFLP, as well as the cell survival after UV irradiation. In this paper, we describe our experience of XP-A prenatal diagnosis for 10 pregnant patients, who had at least one XP-A child as a proband, from nine unrelated Japanese families.

METHODS

XP-A families

All of the families examined are listed in Table 1. All of the probands were determined to have XP-A and were homozygous for IVS3-1G>C. Their parents, who were both genetic carriers of XP-A, were not consanguineous. When a mother became pregnant, she was referred to our department for the prenatal tests for XP-A. We provided genetic counseling for the parents of all probands and obtained informed consent for the prenatal analysis for the diagnosis of XP-A, in accordance with the guidelines of the local institutional ethical committee board and the principles of the Declaration of Helsinki. Informed consent was obtained under a Hamamatsu University School of Medicine (families 2–3) – or Osaka Medical College (families 4–9) – review board-approved protocol.

The client from family 1 was our first trial case for XP-A prenatal diagnosis in 2000 and this examination was performed before the establishment of an ethical committee at Hamamatsu University School of Medicine in 2001. We obtained samples of both chorionic villi tissue and AF by mail from Tokyo Medical and Dental University. All of the cases from the families 2–9 were performed by amniocentesis at one of our two institutions.

Chorionic villus sampling (CVS) and amniocentesis (AM)

The CVS for family 1 and the AM for all of the families were performed at 10 and 14–16 weeks of pregnancy, respectively, under spinal anesthesia (CVS) and local anesthesia (AM) without any problems during these procedures. However, 6 weeks after the CVS and 4 days after taking the AF from the fetus in family 1, forewaters were formed and *in utero* fetal death occurred due to an unknown cause without any signs of intrauterine infection. The dead fetus was removed artificially.

Amplification of the XPA gene by PCR and AlwNI RFLP

Genomic DNA was extracted from the CV tissue, peripheral blood leukocytes derived from parents, cells in AF and cultured cells (N-3, XP24HM and cultured amniotic cells) using a QiaAmp DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. A genomic DNA fragment containing exon 4 with 5′- and 3′-flanking introns of the *XPA* gene was amplified by PCR using primers as follows: sense primer 5′-GGGAATTCTTGCTGGGC-TATTTGCAAAC-3′ and anti-sense primer 5′-GGGGATCCGCCAAA-CCAATTATGACTAG -3′[7] and EX Taq DNA Polymerase (Takara Bio, Shiga, Japan). The PCR steps consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and elongation at 72°C for 1 min. The amplified fragments were digested with *AlwNI* (New England Biolabs, Beverly, MA, USA), and were separated by electrophoresis in 2% agarose gel and stained with ethidium bromide. Next, the 328-bp PCR product was generated and digested by *AlwNI* for 3 h at 37°C resulting in two fragments (84 and 244 bp) if a new cleavage site for *AlwNI* was created by a G>C substitution (IVS3-1G>C) in the *XPA* gene.

Finally, we examined the *XPA* gene of DNA extracted from the newborn infants and reconfirmed the genotype of *XPA*, except the case of family 1 and the second case from family 7.

Cells and cell culture

Amniotic fluid (5 mL) was added to 5 mL of 2 × Dulbecco's minimum essential medium (DMEM; Sigma Chemical, Tokyo, Japan) with 20% fetal bovine serum (FBS; Filtron, Altona, Australia) and

Table 1. List of families undergoing prenatal diagnosis for XP-A by chorionic villi sampling (case 1) and amniocentesis (all cases)

Case no.	XP-A family no.	Age of father (years)	Age of mother (years)	Age of proband at visit (years)	*XPA* mutation in proband	Sample taken for analysis (weeks of pregnancy)	Result of prenatal analysis (final diagnosis and course)
1	1	32	30	XPA (first child, 3 years)	IVS3-1G>C, homozygous	CV (10 weeks), AF (15 weeks)	IVS3-1G>C, heterozygous IVS3-1G>C (XP-A carrier, intrauterine death)
2	2	41	37	Three XPA children (11, 7, 2 years)	IVS3-1G>C, homozygous	AF (16 weeks)	IVS3-1G>C, homozygous (XP-A, born)
3	3	36	33	XPA (second child, 2 years)	IVS3-1G>C, homozygous	AF (15 weeks)	IVS3-1G>C, heterozygous (XP-A carrier, born)
4	4	36	33	XPA (first child, 2 years)	IVS3-1G>C, homozygous	AF (14 weeks)	No mutation (wild type, born)
5	5	35	32	XPA (first child, 3 years)	IVS3-1G>C, homozygous	AF (14 weeks)	IVS3-1G>C, heterozygous (XP-A carrier, born)
6	6	34	31	XPA (first child, 3 years)	IVS3-1G>C, homozygous	AF (14 weeks)	No mutation (wild type, born)
7	7#	33	32	XPA (first child, 2 years)	IVS3-1G>C, homozygous	AF (14 weeks)	IVS3-1G>C, heterozygous (XP-A carrier, born)
8	8	33	30	XPA (first child, 1 year)	IVS3-1G>C, homozygous	AF (15 weeks)	IVS3-1G>C, heterozygous (XP-A carrier, born)
9	9	34	29	XPA (first child, 2 years)	IVS3-1G>C, homozygous	AF (15 weeks)	IVS3-1G>C, heterozygous (XP-A carrier, born)
10	7#	36	35	XPA (first child, 5 years)	IVS3-1G>C, homozygous	AF (15 weeks)	IVS3-1G>C, homozygous (XP-A, abortion)

#, same family, the mother in this family experienced XP-A prenatal diagnosis twice; CV, chorionic villi; AF, amniotic fluid.

antibiotics (100 units/mL of penicillin G and 100 μg/mL of streptomycin; Gibco, Gaithersberg, MD, USA). The cells were cultured for 5 days in an incubator, then the attached monolayer cells were grown and cultured until genetic and cell biological analyses. N-3 and XP24HM, primary human fibroblast cell lines derived from a healthy subject (a 3-year-old Japanese male) and a XP-A patient (6-month-old Japanese female) with the homozygous *XPA* mutation (IVS3-1G>C), respectively, were previously established in our laboratory and were used as controls in the present analysis.[11] We also used primary cultured fibroblasts from the proband's skin from each XPA family. All of the cells were maintained in DMEM with 15% FBS and antibiotics at 37°C in a 5% CO_2 atmosphere.

UV treatment and post-UV survival

Ultraviolet treatment was performed with a germicidal lamp emitting predominantly 254 nm light (Toshiba GL10, Toshiba Electric, Tokyo, Japan) at a dose of 1.66 J/m^2/s. Fluence rate was measured by a UV radiometer (UVR-2; Topcon, Tokyo, Japan). Cell survival was determined as described previously.[12] In brief, 2×10^4 cells were plated per 35-mm dish and were treated with UV doses of 0–15 J/m^2. Seven days after UV exposure, the cells were stained with crystal violet. Survival was compared between the cells in the UV-treated dishes and the cells in the non-UV irradiated dishes.

RESULTS

All of the cases examined in the present study are listed in Table 1, showing that we examined 10 pregnant patients who had at least one XP-A child as a proband, from nine unrelated Japanese families. So far, we have confirmed the prenatal diagnosis of XP-A by the combination of a PCR-based genetic study and post-UV cell survival in 10 cases from nine unrelated Japanese families. Unfortunately, although the fetus was successfully diagnosed as an XP-A carrier, the fetus in the first family died *in utero* after CVS and AM, the reason for which remains unknown. We decided to perform prenatal diagnosis using only amniocentesis in the subsequent cases (from families 2–9).

We have so far successfully prenatally identified two XP-A cases, 5 XP-A carriers and two fetuses with a wild-type *XPA* gene. From family 2, there were no deleterious effects during or after the AM procedure. The results of a representative case are presented below.

As shown in Figure 1(a), a PCR-amplified and *AlwNI*-treated genomic DNA sample containing exon four with 5′- and 3′-flanking introns of the *XPA* gene from AF in family 8 generated a 244-bp band as well as the 328-bp band. The 84-bp band was not visible because of the low sensitivity to detect it using the agarose gel system instead of polyacrylamide gel system.

In spite of the inability to detect the 84-bp band, we have been using agarose gel electrophoresis because it provides an easy and quick system, and a prenatal diagnosis should be obtained as soon as possible. DNA from N-3 cells gave only one band of 328 bp, while DNA from XP24HM cells gave both the 328-bp and 244-bp bands, although the 84-bp band was undetectable. As shown in Figure 2(b), bands of 328 and 244 bp appeared in the PCR products from the cells cultured from the AF. These data suggest that the fetus was likely a heterozygote for a IVS3-1G>C mutation in the *XPA* gene.

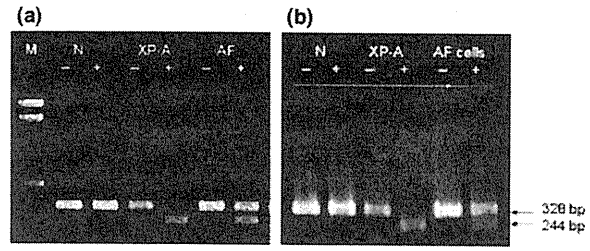

Figure 1. Results of the polymerase chain reaction (PCR) restriction fragment length polymorphism analysis of the *XPA* gene in amniotic fluid AF (a) and the cultured AF cells (b) in family 8. (A) PCR-amplified, *AlwNI*-treated DNA fragments of AF, XP24HM cells (XP-A) and N-3 cells (N) are represented. (B) PCR-amplified, *AlwNI*-treated DNA fragments of the cells from the cultured AF cells, XP24HM (XP-A) and N-3 (N) cells are depicted. M, DNA marker of 100-bp ladder; bp, base pairs; +, *AlwNI* treated; –, *AlwNI* untreated.

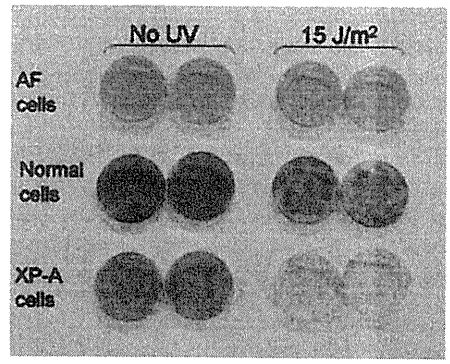

Figure 2. Survival of cells after ultraviolet (UV) from the amniotic fluid (AF) of the case from family 8. The sensitivity to killing by UV in the cultured AF cells was comparable to that in N-3 cells (N), while the survival of XP24HM cells (XP-A) was extremely low.

We next analyzed the sensitivity of the cultured AF cells to UV and observed similar sensitivity to killing by UV compared to normal cells. As shown in Figure 2, the sensitivity to killing by UV in cultures AF cells was almost equal to that of normal (N-3) cells. The survival of XP24HM (XP-A) cells was extremely low in the same experiment.

After checking both the genetic and cell culture findings, we diagnosed the prenatal case from family 8 as an XP-A carrier. We again examined the *XPA* gene after the birth of the infant and confirmed the case to be an XP-A carrier.

DISCUSSION

Recent progress in molecular biology and genetics has been making it possible to perform prenatal diagnosis for various genetic diseases during the early stage of the pregnancy. There are two major procedures to take specimens from a fetus. One is AM and the other is CVS (Table 2). The method most often used for the prenatal diagnosis has been AM, although it is necessary to wait until 14 weeks of pregnancy to obtain the AF. Because it takes an

Table 2. Procedures used for the prenatal diagnosis of XP-A

	Chorionic villus sampling	Amniocentesis
Time for examination (weeks of pregnancy)	8–11 weeks	14–17 weeks
Approach	Transabdominal transvaginal	Transabdominal
Technique	Difficult	Easy
Deleterious effects	Infection abortion (4–5%) teratogenesis	Infection abortion (<0.5%)
Artificial abortion	Easy, bearable	Not easy, mentally unbearable
DNA repair studies† (materials and time needed)	Impossible	Possible cultured cells, 2–3 weeks
DNA analysis‡ (materials and time needed)	Possible trophoblasts, 1–3 days	Possible amniotic fluid, 1–3 days cultured cells, 2–3 weeks
Contamination of maternal tissue	Considerable	Negligible

†Ultraviolet (UV) survival, post-UV inhibition of DNA synthesis using cultured cells derived from amniotic fluid; ‡polymerase chain reaction restriction fragment length polymorphism analysis.

additional 2–3 weeks to finish the DNA repair studies, such as the measurement of UV survival or post-UV inhibition of DNA synthesis using a cell line established from AF, this leads to a relatively late diagnosis. In contrast, by CVS, it is possible to do the chromosomal and DNA analyses and judge the fetal abnormality at 8 weeks of pregnancy. Although CVS is useful to detect the molecular abnormalities by PCT-RFLP in a fetal case of possible XP-A at the early stage of the pregnancy, there are several disadvantages: (i) the amount of tissue available from chorionic villi is limited; (ii) there is an increased risk of spontaneous miscarriages, neonatal death and teratogeny by performing CVS; and (iii) there is a potential for maternal contamination by taking chorionic villi, which may cause the misdiagnosis of XP in genetic analysis by PCR. When we take AF, there is a possibility of the contamination of maternal cells such as white blood cells. So we usually perform UV survival assay to confirm the phenotype of a fetus in addition to DNA analysis by PCR-RFLP. UV survival assay is easier than the measurement of post-UV UDS and Western analysis for XPA protein.

In the present study, we first diagnosed a fetus (family 1) as a carrier of XP-A by the PCR-RFLP technique using chorionic villi at 10 weeks of pregnancy. In order to avoid the misdiagnosis due to the possibility of the mixture of maternal cells at the sampling of chorionic villi tissue, we next performed amniocentesis 5 weeks after the CVS. Although the precise cause of the intrauterine fetal death after the CVS and AM remains unclear, the use of these two procedures in a short period might have impacted the survival of the fetus *in utero*. It has been demonstrated that AM is safer than CVS, and the benefits of an earlier diagnosis by CVS must be weighed against its greater risk. Our unexpected experience presented here suggests that the combination of the two major procedures, CVS plus AM, for the prenatal diagnosis of XP should be carefully performed to ensure neonatal and maternal safety. We therefore decided to perform only AM for XP-A prenatal diagnosis beginning from family 2. So far, there has been no adverse events for any of the pregnant mothers or fetuses from case 2 through case 10.

We have now examined 10 prenatal cases from the nine unrelated XP-A families and have confirmed the diagnosis of two XP-A cases, six XP-A carriers and two subjects with the wild-type *XPA* gene, which seems to be consistent with Mendel's law.

Among the eight types of XP, patients with groups A, B, D and G of XP may have progressive neurodegenerative symptoms. In Japan, XPA is not a rare disease and the frequency of XP-A heterozygotes is 0.88%,[10] which is higher than in most other countries; in addition, there is a strong hot spot for the mutation of the *XPA* gene in Japanese XP-A patients, resulting in a rapid and easy detection of genetic changes of the *XPA* gene. Based on these findings, the genetic counseling and medicine related to XP, especially XP-A, will continue to be needed in Japan, although the prenatal diagnosis for XPA needs close attention ethically, technically and psychologically for XP-A family members.

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unscheduled DNA synthesis

機能的脳神経外科

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脳深部刺激療法施行後長期経過を観察しえたパントテン酸キナーゼ関連神経変性の一例

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磯尾 綾子[3]／川崎 隆[3]／木村 活生[2]／青木 寧子[2]／浜田 生馬[4]

抄　録：パントテン酸キナーゼ関連神経変性（PKAN）は，パントテン酸キナーゼ2遺伝子変異による常染色体劣性遺伝性疾患であり，淡蒼球・黒質への鉄沈着を特徴とする。小児早期より進行性の全身性ジストニアを生じ，内科的治療に抵抗性である。近年，これに対する淡蒼球内節刺激術（GPi-DBS）の有効性が報告されているが，長期経過の報告はなお少ない。我々は今回，PKAN症例に対してGPi-DBSを施行し，術後7年8ヵ月の経過を観察したので報告する。
症例は14歳，男児。4歳より右上肢のジストニアを生じ，6歳で全身性ジストニアのため臥床状態となった。特徴的なMRI所見よりPKANと診断。7歳で当院にて両側GPi-DBSを施行。ジストニアは著明に改善し，座位保持や寝返りでの移動が可能となった。しかし術後4年3ヵ月より脳神経領域と体幹を主とするジストニアが増悪し，再び臥床状態となる。頭部MRIにてGPi電極の後下方への偏移が認められたため，術後6年で電極再留置術施行。その後初回手術時には及ばないが症状の改善が得られている。
本例におけるDBSの効果減弱には，原疾患の進行に加え，電極位置の偏倚も関係していたと考えられる。本例では，成長に伴う脳の発達とともに，原疾患に伴う脳の萎縮があり，このアンバランスにより電極位置が偏倚した可能性を考えた。小児および神経変性疾患に対する脳深部刺激療法の問題点を考える上で示唆に富む症例と考えた。

索引用語：パントテン酸キナーゼ関連神経変性；脳深部刺激療法；長期予後；電極偏倚；小児

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機能的脳神経外科 50(2011)180-184

パントテン酸キナーゼ関連神経変性（PKAN）は，淡蒼球・黒質への鉄沈着を特徴とし，小児早期より進行性の全身性ジストニアを生じる常染色体劣性遺伝性疾患である。近年本疾患のジストニアに対する淡蒼球内節刺激術（GPi-DBS）の有効性が報告されているが，術後の長期経過を追跡した報告は少ない。今回，PKAN症例に対するGPi-DBS後7年8ヵ月の経過を報告する。

症　例

【症　例】
14歳，男児

【家族歴】
母方には特記事項無し。父とは離別し詳細不明。同胞2人は健常。

【発達歴】

周産期に問題を認めず，3080 g，満期産にて出生。乳児早期の運動発達は正常で，1歳すぎより独歩を獲得したが，しばしば頭から後方へ転倒していた。精神発達には遅れを認め，言語は簡単な単語のみであった。

【現病歴】

4歳頃より右上肢を肩から振り回すような不随意運動が出現。不随意運動は次第に全身に広がり，さらに頸部を後屈させるジストニア姿勢も見られるようになった。5歳より下肢のジストニアが増悪し歩行不能。前医にて特徴的な頭部MRI所見よりPKANと診断された。トリヘキシフェニジル，ジアゼパムなど種々の投薬を受けるが効果乏しく，6歳より後弓反張位をとるようになり座位不能。嚥下障害に対して経胃管栄養が開始された。6歳後半では，覚醒時には常に後弓反張位を呈し，痛みを伴う全身の不随意運動を抑制するため終日鎮静剤で眠らせている状態となった。緊張亢進に伴い頻回の嘔吐を認めた。7歳時，GPi-DBSを目的に当院に入院した。

【入院時現症 (Fig.1-a)】

身長107 cm，体重16 kg，BMI 14とるいそうを認めた。全身性ジストニアのため臥床状態で，抱水クロラールによる鎮静でうとうとしているか，疼痛のために啼泣している，という状態であった。脳神経領域のジストニアとして，開口，挺舌，頸部の後屈および右への回旋を認め，喉頭ジストニアに伴う喘鳴を聴取，嚥下障害のため経胃管栄養が施行され，発語は消失していた。体幹は後弓反張位，上肢は屈曲，下肢は内反，随意運動不能であった。下肢を内外転させる激しいジストニア運動が認められた。深部腱反射は減弱していた。入院時のBurke-Fahn-Marsden Dystonia Rating Scaleは，dystonia movement scale(BFMDR-M) 112.5/120点，disability scale (BFMDR-D) 30/30点であった。

【検査所見】

一般採血所見には銅・鉄代謝を含み異常を認めなかった。

眼底検査にて網膜色素変性の所見を認めた。

頭部CTにて両側淡蒼球に高吸収域を認めた

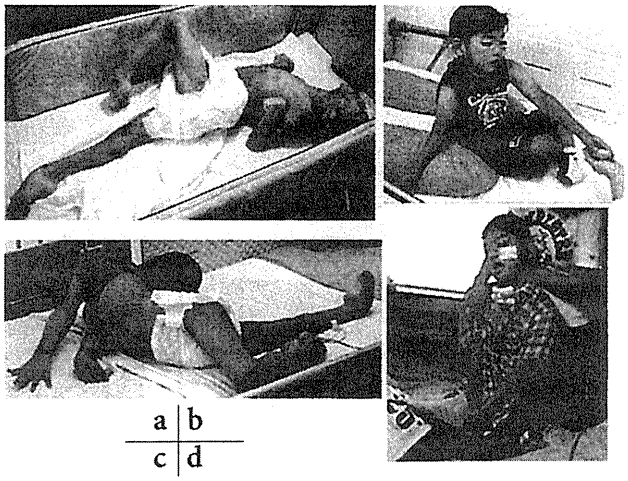

Fig.1 Patient's photographs at 7 years of age (pre-surgery) (a), 3.5 years after surgery (b), 5 years and 4 months after surgery (c), and 1 year and 8 months after lead replacement (7 years and 8 months after the first surgery) (d).

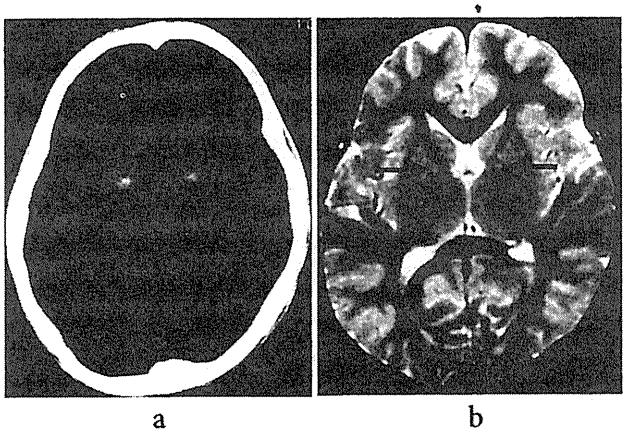

Fig.2 High density areas in the bilateral globus pallidus on brain CT (a), and the eye-of-the-tiger sign (arrows) on brain MRI.

(Fig.2-a)。頭部MRIでは，大脳・基底核・小脳の軽度萎縮に加え，T2強調像にて両側淡蒼球が低信号を示し，この前内側部に高信号域が認められた（the eye-of-the-tiger sign (Fig.2-b))。ECD-SPECTでは両側基底核の血流増加が認められた。

現在パントテン酸キナーゼ2遺伝子(PANK2)変異について検索中である。

【経　過】

臨床経過，網膜色素変性症，特徴的な頭部MRI所見より，PKANと診断。7歳より両側GPi-DBSを開始した(Medtronic 3387電極：Fig.3-a)。刺激開始後速やかに体幹の後弓反張が軽減，ジストニア運動も減少し，抱水クロラールによる鎮静は不要となった。その後もジストニアは経時的に改善。術後10ヵ

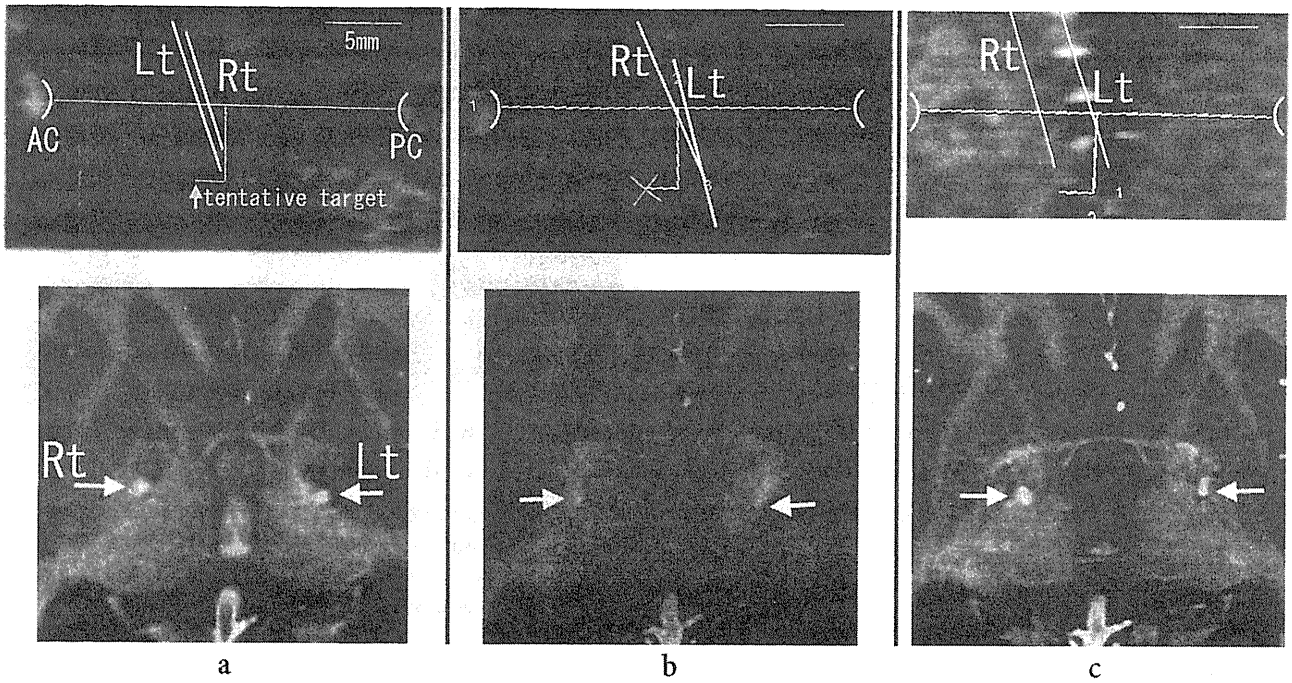

Fig.3 Sagittal (upper panels) and axial (lower panels) planes of brain MRI showing the positions of GPi electrodes. One month (a) and 5 years (b) after the first surgery, and one month after lead replacement (c).
AC: anterior commissure, PC: posterior commissure.

月には座位保持可となり，1年後よりは食事の経口摂取と寝返りが可能となった。術後3年6ヵ月の検査入院時には（Fig.1-b），ジストニアは随意運動時には誘発されるが安静時にはほとんど見られなかった。食事はすべて経口摂取し，単語の発語が見られた。上肢ではじゃんけんや物の把握が可能となり，足関節の拘縮のため立位・歩行はできないが一人で座位をとり寝返りで移動していた。BFMDR-M は 51/120 点に改善した。BFMDR-D は知的障害の影響もあり 25/30 点であった。その後も自宅で同様の状態で生活していた。

しかし，術後4年3ヵ月より，脳神経領域と体幹優位のジストニアが再増悪。刺激の調整を試みるが改善せず，術後5年4ヵ月にはほぼ術前の状態に戻り（Fig.1-c），BFMDR-M 110.5/120 点，BFMDR-D 30/30 点となった。ただし，症状の主体はジストニア姿勢で，術前のように激しいジストニア運動は認められなかった。原疾患の進行に伴う症状の再増悪と考えていたが，頭部 MRI にて GPi 電極の後下方への偏倚（Fig.3-b）が判明。これが刺激効果減弱に関係していると考えた。MRI 上の視察では，大脳・基底核・小脳萎縮の程度は初診時と著変なかった。術後5年4ヵ月で GPi 電極の入れ替え術を行ったが，術後感染のため抜去。電極抜去後喉頭ジストニアがさらに悪化し，気管切開術が施行された。頭蓋内感染の鎮静を待ち，初回術後6年で両側 GPi 電極再留置術を施行した（Fig.3-c）。

現在再留置術後1年8ヵ月（初回術後7年8ヵ月：Fig.1-d）。後弓反張位を伴う体幹の緊張は改善し，支え座位が可となった。四肢のジストニアも改善し，物の把握や下肢の屈伸が可能である。BFMDR-M は 69.5/120 点に改善した。ただし，独座や寝返りはできず，気管切開・経胃管栄養からの離脱もできていない。BFMDR-D は 30/30 点である。

考　察

PKAN は，“脳への鉄沈着を伴う神経変性症”（Neurodegeneration with brain iron accumulation: NBIA）の一つで，PANK2 遺伝子変異による常染色体劣性遺伝性疾患である[4]。淡蒼球・黒質への鉄沈着を特徴とし，小児期より進行性の全身性ジストニアを生じる。ジストニア以外には固縮・舞踏アテトーゼ・振戦などの錐体外路症状を認め，また，錐体路症状，知的障害，構音障害，網膜色素変性症を伴う。頭部 MRI T2 強調像にて，両側淡蒼球が低信号を示し，この前内側部に点状の高信号域を認めることが診断上重要である（the eye-of-the-tiger sign）。淡蒼球全体の低信号域は鉄沈着を，前内側部の高信

号域は浮腫，壊死，グリオーシスなどの神経変性を示すと考えられている。

PKANのジストニアは内科的治療に抵抗性で，運動機能の他，嚥下，呼吸に重篤な障害をもたらす。本例のように発症数年以内に臥床状態となることも多く，生命予後不良である。近年これに対するGPi-DBSの有効性が報告され[1,2,3,6,7,8,9]，PKANはLesch-Nyhan syndromeとともに，DBSの効果の期待されうる代謝変性疾患として注目されている。しかし，術後の長期経過の報告は未だ少なく，4年以上の経過は2論文の3症例で報告されているのみである[1,7]。Krauseら[7]は，13歳時にGPi-DBSを施行したPKAN症例5年の経過を報告した。術前は臥床状態であったが，術後半年で立位保持や数歩の独歩が可能となった。しかし術後1年よりジストニアが再び増悪しはじめ，術後2年で独立・独歩不能，以後も症状は徐々に悪化した。BFMDRS-Mは，術前92，術後1年30，2年40，5年70であった。Adamovicovaら[1]はPKANの兄妹例に対して，それぞれ17歳と16歳でGPi-DBSを施行，術後4.5年および4年の経過を報告した。兄妹ともに術後早期よりジストニアの改善を認めたが，兄は術後18ヵ月より，妹は術後3ヵ月よりジストニアの再増悪をきたしている。兄のBFMDRS-Mは，術前77.5，3ヵ月後15，2年後22.5，4.5年後38。妹のBFMDRS-Mは，術前72，3ヵ月後42.5，2年後53，4年後52であった。2論文とも，PKANにおけるGPi-DBSの効果は原疾患の進行に伴い減弱していくが，術後4〜5年を経過してもなお術前に比べれば有意の改善を認めているとして，GPi-DBSを有効な治療法として推奨している。なお，Krauseらは効果減弱時にGPi電極が偏倚していないことをMRIにて確認しているが，Adamovicovaらの論文には電極偏倚についての記載はない。

本例ではPKANに対するGPi-DBS後7年8ヵ月と，今までで最長の経過を追跡しえた。術後4年3ヵ月までは著明な効果が持続したが，その後脳神経領域と体幹優位のジストニア姿勢が急激に増悪，術後5年4ヵ月でほぼ術前同様の状態となった。ただし術前に見られた下肢の激しいジストニア運動は再燃しなかった。MRIにてGPi電極の後下方への偏倚が明らかとなり，電極再留置術により症状は初回術後ほどではないが再び改善した。

本例ではさきの2論文の3症例よりも長期にわたりDBSの効果が持続した。PKANの重症度には症例による差があるが，本例は発症後急激にジストニアが進行して2年で臥床状態となっており，2論文例より軽症であったとは考えにくい。本例では症状に応じて，論文例よりも頻回の刺激調整が行われており，これが効果の維持に関係した可能性がある。

ジストニアに対するGPi-DBSでは，術後早期にジストニア運動が消失し，ジストニア姿勢は遅れて経時的に改善していく[11]。GPi-DBSの効果減弱の過程を観察しえた本例では，ジストニア姿勢のみが再増悪し，ジストニア運動は再燃しなかった。Krauseら[7]のPKAN症例においても同様の経過が記載されている。ジストニア運動とジストニア姿勢に対するGPi-DBSのこのような効果の違いは，両者の発現系の違いを反映する可能性があり，興味深い。

本例におけるジストニアの再増悪には，原疾患の進行に加え，電極位置の偏倚が関与していたと考えられる。Yianniら[10]は，ジストニア症例に留置したDBS電極の3.9%（3/76本）に後に偏倚（slip）を生じた，と報告した。このような偏倚はパーキンソン病・振戦など他の運動異常症の症例に留置した164の電極には認められず，体軸のジストニアによる牽引のために電極や固定具に負荷がかかるものか，と推測している。ただし彼らの症例では電極偏倚は術後約1年で生じており，使用した固定具や，電極と刺激装置をつなぐコードの長さにも問題のあった可能性がある。本例では偏倚の時期はより遅いと考えられ，また推奨される固定具を使用して，コードにも十分なゆるみを与えているので，Yianniらの症例と同様の機序が働いたとは考えにくい。本例では7歳で電極留置術を施行しており，その後の成長に伴い脳の容積は増大している。一方，PKAN症例には軽度ながら大脳萎縮を生じることが報告され[5]，本例にも大脳・基底核・小脳の萎縮が見られている。小児期における脳の発達と，原疾患による脳萎縮のアンバランスが，電極位置の相対的偏倚をもたらした可能性を考えた。本例のMRIの視察では明らかでなかったが，電極が後下方に偏倚したことから大脳萎縮は前頭葉優位に進行したことが推測される。小児期に電極留置術を受けた症例，特に神経変性疾患の症例においては，長期経過中に成人例とは異なる問題を生じてくる可能性があり，今後の症例の集積と詳細な検討が必要である。

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Long-term outcome of bilateral pallidal stimulation in a child with pantothenate kinase-associated neurodegeneration

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Abstract: Pantothenate kinase-associated neurodegeneration (PKAN) is a rare autosomal recessive disorder characterized by excessive iron accumulation in the globus pallidus and the substantia nigra. Early-onset progressive generalized dystonia represents a major clinical feature of PKAN, which is refractory to pharmacological therapies and often causes severe disability and fatal outcome. Although several recent reports suggest a beneficial effect of deep brain stimulation of the bilateral internal globus pallidus (GPi-DBS), its long-term outcome is unclear. We present a 7-year and 8-month follow-up in a child with PKAN treated by GPi-DBS.

The boy developed dystonic movements in his right arm at the age of 4 years. Dystonia rapidly progressed to be generalized and he became bedridden with opisthotonic posture at age 6. Violent involuntary movements were observed in his extremities. Pharmacological therapies were ineffective. He was diagnosed as PKAN by a specific pattern on brain MRI called the eye-of-the-tiger sign, which is pathognomonic for the disease. Bilateral GPi-DBS was started at age 7. The opisthotonic posture and involuntary movements remarkably decreased soon after surgery. His motor function gradually improved. He could sit unsupported after 10 months and roll over after 1 year. The Burke-Fahn-Marsden's Dystonia Motor Score (BFMD-M) improved from 112.5 points before surgery to 51 points 3 years and 6 months after surgery. His condition remained stable for 9 months further. However, progression of dystonia predominating in the oromandibular, cervical, and truncal regions followed, and he returned to a bedridden status 5 years and 4 months after surgery. The BFMD-M deteriorated to 110.5 points. Comparison of brain MRIs at that time with those performed 1 month after surgery disclosed postero-inferior dislocation of the bilateral GPi electrodes. Lead replacement was conducted 6 years after the first surgery. His condition gradually improved, although his motor function did not reach the best level after the first surgery. The BFMD-M was 69.5 points 1 year and 8 months after the lead replacement.

This is the longest follow-up of a case of PKAN treated by GPi-DBS. A remarkable efficacy persisted for 4 years, but his motor function deteriorated during subsequent years. The dislocation of the GPi electrodes in addition to disease progression caused the decrease in DBS efficacy. Imbalance between cranial growth during early childhood and brain atrophy observed in PKAN may be related to the lead dislocation in our patient.

Keywords: Pantothenate kinase-associated neurodegeneration; Deep brain stimulation; Long-term outcome; Lead dislocation; Childhood

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Extracellular Recombinant Annexin II Confers UVC-Radiation Resistance and Increases the Bcl-xL to Bax Protein Ratios in Human UVC-Radiation-Sensitive Cells

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In this study, we found that refractoriness to ultraviolet (UVC) light-induced cell death was increased in UVC-radiation-sensitive cells derived from Cockayne syndrome patients when the cells were precultured in medium supplemented with recombinant annexin II (rANX II). In CS3BES cells, an immortal cell line derived from Cockayne syndrome patients, the rANX II supplementation-induced UVC-radiation resistance was suppressed by treatment with an anti-annexin II antibody and EGTA. The amount of biotinylated annexin II on the cell surface increased in the rANX II-supplemented cells but did not increase in the cells that were cotreated with rANX II and EGTA. The capacity to remove UVC-radiation-damaged DNA, (6-4) photoproducts and cyclobutane pyrimidine dimers, was the same in cells that were precultured with rANX II and in control cells that did not receive rANX II supplementation. The rANX II supplementation-induced UVC-radiation resistance was also observed in nucleotide excision repair-deficient cells and xeroderma pigmentosum group A-downregulated cells. The Bcl-xL to Bax protein ratios, an index of survival activity in cells exposed to lethal stresses, were increased in the cells that had been precultured in rANX II for 24 h prior to UVC irradiation. Treatment with a phosphatidylinositol 3-kinase inhibitor suppressed the increased UVC-radiation resistance and Bcl-xL to Bax ratios in the cells with rANX II supplementation. Furthermore, downregulation of Bcl-xL by siRNA transfection also suppressed the UVC-radiation resistance that was induced by rANX II supplementation. These results suggest that the increase in the Bcl-xL to Bax ratios may be associated with enhanced resistance to UVC-radiation-induced cell death. © 2011 by Radiation Research Society

INTRODUCTION

Cockayne syndrome (CS) is a rare autosomal recessive disease characterized by acute sun sensitivity, cachectic dwarfism, skeletal abnormalities, pigmentary retinal degeneration, and progressive neurological defects including dementia (*1*). Cultured cells from CS patients (CS cells) are hypersensitive to the cell-killing effects of radiation, particularly 254-nm wavelength ultraviolet light (UVC radiation) (*2*). The molecular mechanisms underlying this hypersensitivity have been studied extensively, and the details of the abnormalities in DNA metabolism in CS cells have been clarified (*3*). However, there is no proposed method to reduce the cellular UV-radiation hypersensitivity effectively in the cells of CS patients.

UVC radiation does not reach the Earth, while UVB radiation (280–320 nm) and UVA radiation (320–400 nm) do. UVC and UVB radiation cause dimeric pyrimidine damage to DNA, and UVB and UVA radiation cause oxidative damage to DNA. In our study, we used UVC radiation as a DNA-damaging agent to elucidate responses of human cells to the DNA-damaging effects of UV radiation. Human cells may possess common mechanisms to protect against the DNA-damaging effects of UVB and UVC radiation, and we recently determined that a cellular protective mechanism against UVC radiation can also function to protect human cells from UVB radiation (unpublished data).

We previously found that CS fibroblasts that were cultured in medium containing human interferon (HuIFN)-β and subsequently irradiated with UVC radiation exhibit

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increased colony formation (*4*). By studying the mechanisms that underlie the HuIFN-induced UVC-radiation resistance, we recently identified chaperone proteins, such as heat-shock protein (HSP) 27, that are involved in this resistance (*5*). Additionally, we found that one of the HSP27-binding proteins, annexin II, is also involved in UVC-radiation resistance (*6, 7*).

Annexin II belongs to a family of calcium-dependent, phospholipid-binding proteins that are expressed in a diverse range of tissues and cell types (*8*). Initially identified as an intracellular molecule, annexin II has been implicated in the regulation of a variety of cellular processes, including exocytosis (*9*) and endocytosis (*10*). In addition to its intracellular functions, annexin II is also secreted into the extracellular environment in both soluble and membrane-bound forms (*11*). Although the functions of extracellular annexin II are not fully understood, annexin II is known to act as a cell surface receptor for extracellular ligands and has possible roles in the regulation of proteolytic cascades (*12*), signal transduction (*13*) and tumor invasion and metastasis (*14–17*). After the loss of anchorage (anoikis), a protective role of extracellularly supplemented annexin II against apoptosis has been reported (*18*). However, there are no reports investigating the ability of extracellular annexin II to modulate the survival capacity of human cells after UVC irradiation, particularly in cells with a high susceptibility to UVC-radiation-induced cell death. It is important to elucidate the roles of extracellular annexin II that can confer resistance to UVC radiation to human cells that are sensitive to UV radiation and the molecular mechanisms underlying these as a means to reduce cellular hypersensitivity to UV radiation in the cells of CS patients.

In this study, we examined whether extracellularly added recombinant annexin II (rANX II) affects the survival capacity of CS cells after UVC irradiation. We found that the cells precultured in medium supplemented with rANX II exhibited an increased resistance to UVC-radiation-induced cell death.

MATERIALS AND METHODS

Cells and Culture Conditions

Primary cultured cells from Cockayne syndrome (CS) patients, CSBB (*19*), and two immortal cell lines derived from CS patients, CS1AN-S3-G2 (CS1ANS) and CS3BE-S3-G1 (CS3BES) (*20*), were cultivated. CS1ANS and CS3BES have defective CS group B (CSB) and CS group A (CSA) genes, respectively, and were provided by Dr. A. R. Lehmann. The two CS cell lines were immortalized with the pSV3gpt plasmid, which contains the Simian virus 40 (SV40) early region encoding the T antigen and the bacterial gene xanthine-guanine phosphoribosyl transferase. UVC-radiation-sensitive human RSa cells, which were established from human embryo-derived fibroblastic cells by double transfection with SV40 and Rous sarcoma virus (*21*), human AP^r-1 cells, which were established from RSa cells by mutagenesis with ethyl methanesulfonate followed by UVC irradiation (*22*), and HeLa cells, which are a cervical cancer cell line (*23*), were used. The sensitivity of AP^r-1 cells and HeLa cells to UVC radiation is similar to and lower than that of normal human fibroblasts, respectively (*24*, our preliminary results).

XP6BES and XP2OS, two immortal cell lines derived from xeroderma pigmentosum (XP) patient, were also used (*25*). These two cell lines were immortalized by infection with SV40 and have defective XP group D (XPD) and XP group A (XPA) genes, respectively. The CSBB cells were cultured in Eagle's MEM (EMEM; Nissui, Tokyo, Japan) containing 10% (v/v) fetal bovine serum (Hyclone Laboratories, Inc.), and the other cells, including the immortalized cell lines from CS and XP patients, were cultured in EMEM containing 10% (v/v) calf serum (Gibco Invitrogen Corp., Grand Island, NY). All cells were cultured at 37°C in a humidified atmosphere containing 95% air/5% CO_2.

UVC Irradiation

UVC irradiation (200–280 nm) was performed as described previously (*5*). The intensity of the radiation was 0.4 $J/m^2/s$ for CS and XP cells and 1.0 $J/m^2/s$ for the other cells. The intensity was measured by a UVR-254 UV radiometer (Tokyo Kogaku Kikai Co., Tokyo, Japan). Mock-irradiated cells were treated in the same manner but were not irradiation.

Preparation of Recombinant Annexin II

Full-length human annexin II cDNA was prepared as described previously (*6*). The cDNA was fused to the 3′ end of the glutathione S-transferase (GST) gene in the pGEX-6P-1 vector plasmid (GE Healthcare UK Limited, Buckinghamshire, UK) using *Bam*HI and *Xho*I restriction sites. Expression of GST-fused annexin II (GST-ANX II) was induced by IPTG in *Escherichia coli* (*E. coli*) with XL-1 Blue. *E. coli* pellets expressing the proteins were lysed by sonication in a buffer containing PBS (pH 7.4), 1 m*M* dithiothreitol (DTT), 0.5 m*M* EDTA, 1.0% Triton X-100 and protease inhibitors including 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.005 m*M* leupeptin, 0.005 m*M* pepstatin A, and 0.005 m*M* E64. Next, GST-ANX II in the lysates was bound to a glutathione (GSH) Sepharose 4 Fast Flow (GE Healthcare) column, and the recombinant annexin II (rANX II) was separated from the GST-ANX II by PreScission protease (GE Healthcare) digestion and eluted from the column according to the manufacturer's recommendations. GST was also eluted from the column after elution with glutathione and used as a control for the rANX II supplement. Eluates from the column were dialyzed against 20 m*M* Hepes, pH 7.4, containing 0.1 *M* NaCl, 10% glycerol, 0.2 m*M* DTT, and 0.5 m*M* PMSF, sterilized with a 0.20-μm filter, and then frozen at −80°C in small aliquots. Purity of the rANX II sample was estimated by CBB staining of an SDS gel followed by quantification of the signal intensity of the protein bands (Supplementary Fig. 1; http://dx.doi.org/10.1667/RR2561.1.S1). The rANX II sample (1.0 mg/ml) contained rANX II (0.97 mg/ml) and a small amount of GST (0.032 mg/ml).

rANX II Treatment

Cells were plated in 60-mm dishes (5×10^5 cells/dish) and incubated for 24 h to allow the cells to attach, and then rANX II was added to the medium at the indicated dose. After culturing in the presence of rANX II for the indicated time, the cells were used for each experiment.

Colony Survival Assay

Cells that were treated with rANX II were harvested and suspended in 0.72 m*M* $CaCl_2$-containing PBS (1.7×10^3 cells/ml). One milliliter of the cell suspension was spread out on 100-mm dishes, irradiated with UVC radiation, and cultured in 10 ml of medium for 2 weeks. Next, colonies were counted, as described previously (*5*). For the inhibitor experiment, LY294002 (50 μ*M*; Sigma-Aldrich Corp. St. Louis, MO), a phosphatidylinositol 3-kinase (PI3K) inhibitor, was added 30 min before the addition of rANX II, and cells were cultured for an additional 24 h in the presence of the inhibitor and rANX II and then harvested and used for the colony survival assay.