DJ-1 degrades transthyretin and an inactive form of DJ-1 is secreted in familial amyloidotic polyneuropathy.
Abstract. DJ-1 plays roles in transcriptional regulation and anti-oxidative stress, and loss of its function is thought to result in the onset of Parkinson’s disease. DJ-1 has a protease-like structure and transthyretin (TTR), a protein causing familial amyloidotic polyneuropathy (FAP), was identified as a substrate for DJ-1 protease in this study. Both TTR and DJ-1 were secreted into the culture medium under normal conditions, and secreted TTR was not aggregated. Under oxidative conditions, TTR but not DJ-1 was secreted into the culture medium, resulting in aggregation. Mirror images of both the expression patterns and solubility of DJ-1 and TTR were observed in tissues of FAP patients, and an unoxidized form of DJ-1, an inactive form, was secreted into the serum of FAP patients. These results suggest that oxidative stress to cells abrogates secretion of DJ-1 and that secreted DJ-1 degrades aggregated TTR to protect against the onset of FAP.

Introduction

Amyloidosis is caused by the deposition of proteins that are normally soluble under physiological conditions (1,2). Transthyretin (TTR) is one of the 23 human proteins known to be associated with local or systemic amyloidosis (3-6). TTR forms a homo-tetramer that carries a retinol-binding protein with retinol and thyroxine in the plasma and cerebrospinal fluid. Senile systemic amyloidosis is a sporadic disorder resulting from the deposition of wild-type TTR fibrils in cardiac and other tissues. Familial amyloidotic polyneuropathy (FAP) and cardiomyopathy, on the other hand, hereditary autosomal-dominant diseases in which deposition of TTR fibrils is caused by amyloidogenic mutations, and these diseases primarily affect the peripheral and autonomic nervous systems and the heart, respectively. Although it is known that TTR deposits are formed extra-cellularly (7), the mechanism by which FAP is caused is not clear. DJ-1 was initially identified by us as a novel oncogene (8) and has recently been found to be a causative gene for familial Parkinson’s disease PARK7 (9). DJ-1 has also been shown to be related to fertilization (10,11) and tumorigenesis (12,13). We and others found that DJ-1 plays roles in transcriptional regulation (14-18), mitochondrial regulation (19) and anti-oxidative stress (20-23), and loss of its function is thought to result in the onset of Parkinson’s disease. In addition to its anti-oxidative stress function, DJ-1 has a structure similar to that of protease I, a cysteine protease of Pyrococcus horikoshii (24). Compared to protease I, DJ-1 contains an additional α-helix (α-helix 9) at the C-terminal region, which blocks the putative catalytic site of DJ-1 and appears to regulate enzymatic activity (24). Biochemical analyses have shown that DJ-1 works as a protease (25) or chaperone (26,27), though controversial results have been reported (28,29). DJ-1 has three cysteines at amino acid numbers 53, 46 and 106 (C53, C46 and C106, respectively). Of the three cysteine residues, C106 is highly sensitive to oxidation compared to the others (32), and oxidation of C106 is essential for DJ-1 to exert its full activities, including anti-oxidative stress function (22,23,33), protease (25) and chaperone (26,27) activities. Regarding its chaperone activity, DJ-1 with C106 oxidized as an SO2H form, but not with C106 reduced or oxidized as an SO3H form, has been shown to be active (27). C53 and C46 have, on the other hand, been shown to be nitrosylated and to be necessary for dimer formation of DJ-1 (28). In addition to the oxidation of DJ-1, the activities of DJ-1 have been reported to be regulated by SUMO-1 conjugation on a lysine residue at amino acid number 130 (K130) (29).

In this study, we found that TTR co-secreted with DJ-1 into culture medium was degraded as a substrate for DJ-1 protease under normal conditions, but that secretion of DJ-1 was abrogated under oxidative conditions, resulting in the formation of amyloid fibrils of TTR. Furthermore, an inactive form of DJ-1 was secreted into the serum of FAP patients.
Materials and methods

Cell culture and transfection. Human 293T and HEK293 cells and mouse Flp-InNIH3T3 (Invitrogen) and D2 cells (33) were cultured in Dulbecco’s modified Eagle’s medium with 10% calf serum. Transfection of plasmid DNAs into mouse and human cells was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions and by the calcium phosphate precipitation method, respectively.

Establishment of cell lines harboring wild-type TTR or a mutant of TTR. Mutagenesis from valine to methionine at amino acid number 30 in TTR was carried out by PCR using the overlap extension method (33). An EcoRI-XhoI fragment containing Flag-tagged wild-type TTR or mutant TTR was inserted into the EcoRI-XhoI sites of pcDNA3. HEK293 cells were transfected with these DNAs and cultured in medium in the presence of 100 μg/ml Zeocin for 14 days. The cells resistant to the drug were then selected, and expression of Flag-DJ-1 was examined by Western blotting with an anti-Flag antibody (M2, Sigma).

Detection of DJ-1 in the serum of FAP patients. Proteins in the serum of FAP patients and normal persons were subjected to immunoprecipitation with an anti-DJ-1 polyclonal antibody. The precipitates were then separated on 15% polyacrylamide gel containing SDS, transferred onto nitrocellulose membranes, and reacted with an anti-DJ-1 monoclonal antibody (9E8) or with an anti-oxidized DJ-1 antibody (3E8), respectively. Proteins were detected after reacting with Alexa680-conjugated anti-mouse IgG or HRP-conjugated anti-human (Fab)2 antibody. The anti-oxidized DJ-1 antibody was established against a peptide containing SO3-conjugated C106 as an immunogen, and characterization of this antibody was described previously (14).

In vivo binding assay. Ten μg of pcDNA3-Flag-WT-TTR and -M30V-TTR was transfected into human 293T cells that were 60% confluent in a 10-cm dish by the calcium phosphate precipitation technique. Forty-eight hours after transfection, ~2 mg of proteins in the whole cell extract was first immunoprecipitated with an anti-Flag antibody (M2, Sigma) as described previously (14). The precipitates were then separated onto a 15% polyacrylamide gel containing SDS and analyzed by Western blotting with anti-DJ-1 (15) and anti-FLAG antibodies. Immunoprecipitated proteins were then reacted with IRDye800 or Alexa680-conjugated anti-mouse IgG or HRP-conjugated anti-human (Fab)2 antibody. The anti-oxidized DJ-1 antibody was established against a peptide containing SO3-conjugated C106 as an immunogen, and characterization of this antibody was described previously (14).

Velocity sedimentation analysis. HEK293 cell lines expressing WT-TTR and M30V-TTR were cultured up to 80% confluence in a 15-cm dish in a serum-free OPTI-MEM medium, and protein extracts were prepared by reacting with a buffer containing 0.5% NP-20, 150 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin and 2 μg/ml pepatatin A. Proteins in the culture medium were precipitated by 60% (NH4)2SO4 and dialyzed against PBS. Proteins in the cell lysates and medium were loaded onto 5-20% (w/v) linear sucrose gradients, centrifuged for 18 h at 4˚C at 35000 rpm in an SW41 rotor, fractionated, and subjected to Western blotting with an anti-Flag or anti-DJ-1 antibody.

Detection of amyloid deposition. Tissue samples were fixed with 10% formalin, embedded in paraffin, serially sectioned at a thickness of 3 mm, and placed onto microscope slides. Sections were stained with hematoxylin and alkaline Congo red and were examined under polarized light in the presence of green birefringence (35,36).

Staining procedures. Slides were treated with 0.3% H2O2 in PBS for 30 min to block endogenous peroxidase activity and incubated in a blocking buffer containing 0.5% bovine serum albumin and 5% goat serum in PBS for 1 h at room temperature in a moist chamber. Slides were reacted with a rabbit anti-human TTR antibody (Dako, Glostrup) diluted (1:100) in PBS, incubated at 4˚C overnight, and washed with PBS. After the slides had reacted with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Dako) diluted 1:100 in the blocking buffer, the reactivity of the proteins was visualized with a DAB liquid system (Dako). Sections were also counterstained with hematoxylin. For parallel control sections, a primary antibody was replaced by the blocking buffer (35,36).

Sandwich ELISA immunoassay procedure. DJ-1 secreted into the serum of FAP patients (V30M) was measured by using Human DJ-1 ELISA kit (CycLex’s, Japan).

Protease activity of DJ-1. A peptide library, FRETS-25Xaa, was obtained from Peptide Institute and used according to the supplier’s manual. FRETS-25Xaa is composed of D2pr(Nma)-Gly-Zaa-Yaa-Xaa-Ala-Phe-pro-Lys(Dnp)-D-Arg-A-Arg, in which Xaa has 19 amino acids lacking cysteine; Yaa is a mixture of Lys, Asp, Tyr, Ile and Pro; and Zaa is a mixture of Arg, Glu, Phe, Val and Ala. After 10 4 M of each amino acid of FRETS-25Xaa was incubated with various amounts of GST-free DJ-1 in a buffer containing 0.5 mM sodium acetate (pH 5.5) and 0.2 mM CaCl2 at 37˚C for 60 min, the highest fluorescence of amino acid of X was determined by measurement of its fluorescence (Ex=360 nm, Em=465 nm) using a fluorescent alimeter.

Results

TRR as a substrate for DJ-1 protease. GST-tagged DJ-1 was expressed in and purified from E. coli, and GST was cleaved off by digestion of GST-DJ-1 by ProScission™ protease (GE Healthcare). To examine the protease activity of DJ-1, recombinant full-sized DJ-1 was reacted with peptide mixtures of FRETS-25Xaa, and the intensity of fluorescence produced by the degradation of the peptides was measured (Fig. 1). The purity of recombinant DJ-1 was >99% as described previously (15). DJ-1 was found to possess protease activity in the presence of 0.1-10 mM Ca2+ (Fig. 1A).
Protease activity of DJ-1 was also found to be active at ~pH 5.5 (data not shown). Full-sized DJ-1 showed weak protease activity, and a C106S mutant of DJ-1, a mutant with a substitution of cysteine to serine at amino acid number 106 corresponding to a catalytic amino acid of protease activity (24,25), had no protease activity (Fig. 1B). DJ-1 (1-174), a truncated form of DJ-1 spanning amino acids 1-174 and lacking α-helix 9, however, showed strong protease activity, and protease activities of both full-sized DJ-1 and DJ-1 (C1-174) were completely lost in the presence of E64, an inhibitor for cysteine protease (Fig. 1B), suggesting that the C-terminal region hindered protease activity of DJ-1 in vitro.

To identify the substrate protein for DJ-1 protease, expression levels of proteins in a DJ-1-knockdown cell line, D2, and its parental mouse NIH3T3 cells (Flp-In-3T3) were compared by two-dimensional gel electrophoresis, and proteins were stained by SYPRO Ruby (Fig. 2). The expression levels of 3 proteins designated by arrows were markedly increased. These protein spots were then broached, digested with trypsin and identified by mass spectrometry (Table I). Three identified proteins were cellular retinoic-acid-binding protein chain A, transthyretin (TTR) and immunoglobulin light chain, and the expression level of TTR was markedly increased. To examine TTR as a target protein for DJ-1 protease, D2 and NIH3T3 cells were transfected with Flag-tagged TTR (F-TTR) and an increased expression level of F-TTR in D2 cells was found by Western blot analysis (Fig. 3A), confirming the results of the proteome analysis. When D2 cells were cotransfected with full-sized wild-type DJ-1 or C106S mutant DJ-1 together with F-TTR, the expression level of TTR was decreased by wild-type DJ-1 but not by C106S mutant DJ-1 (Fig. 3B). Furthermore, wild-type TTR (WT-TTR) and a substitution mutant of TTR (V30M) purified from human tissues were degraded in a dose-dependent manner by recombinant wild-type DJ-1 (1-174) but not by C106S mutant DJ-1 (1-174) in vitro (Figs. 3C and D), indicating that TTR is a substrate for DJ-1 protease. The results also suggest that α-helix 9 in DJ-1 is opened or

Figure 1. Protease activity of DJ-1. GST-DJ-1 and GST-mutants of DJ-1 were expressed in and purified from E. coli as described previously. Purified GST-free DJ-1 and -mutants of DJ-1 were then obtained after digesting GST-DJ-1 and GST-mutants of DJ-1 with PreScission protease to cleave-off GST. (A) Peptide mixtures (10^{-4} M) of FRETS-25Xaa consisting of Gly-(Arg/Glu/Phe/Val/Ala)-(Lys/Asp/Tyr/Ile/Pro)-Arg-Ala-Phe-lys-Arg-Arg were reacted with 1 μg of DJ-1 at 37℃ for 60 min in a buffer containing various concentrations of CaCl₂, and their fluorescences (Ex=360 nm, Em=465 nm) were measured using a fluorescent altimeter. (B) Peptide mixtures (10^{-4} M) of FRETS-25Xaa were reacted with various amounts of DJ-1 and mutants of DJ-1 at 37℃ for 60 min in a mixture as described in Material and methods, and their fluorescences (Ex=360 nm, Em=465 nm) were measured using a fluorescent altimeter.

Figure 2. Comparison of proteins whose expression levels were changed in NIH3T3 and DJ-1-knockdown cells. Proteins were extracted from NIH3T3 (Flp-In-3T3) and its DJ-1-knockdown D2 cells, separated on a two-dimensional gel electrophoresis gel and stained with SYPRO Ruby. Arrows indicate proteins whose expression levels were changed in NIH3T3 and DJ-1-knockdown cells.
cleaved by DJ-1-binding protein(s) in cells, into which full-sized DJ-1 was transfected, to exert protease activity.

To examine the association of DJ-1 with TTR in cells, 293T cells were transfected with Flag-WT-TTR and -M30V-TTR, proteins from transfected cells were immuno-precipitated with an anti-Flag antibody or nonspecific IgG, and the precipitates were analyzed by Western blotting with an anti-DJ-1 antibody (Fig. 4A). The results showed that both Flag-WT-TTR and -V30M-TTR were co-immunoprecipitated with DJ-1 (Fig. 4A, upper part). To examine the

Table I. Identification of proteins whose expression levels were changed in D2 cells by TOF-MS analysis.

<table>
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<tr>
<th>Spot no.</th>
<th>Experimental pI</th>
<th>MW (kDa)</th>
<th>Identified protein</th>
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<td>transthyretin precursor</td>
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<td>3</td>
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<td>25</td>
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Figure 3. Degradation of TTR by DJ-1. (A) NIH3T3 and D2 cells were transfected with expression vectors for Flag-tagged TTR and β-galactosidase. Forty-eight hours after transfection, proteins in transfected cells were analyzed by Western blotting with anti-Flag, anti-mouse DJ-1 and anti-β-galactosidase antibodies as described in Materials and methods. (B) D2 cells were transfected with expression vectors for Flag-tagged full-sized wild-type DJ-1 or C106S mutant DJ-1 together with TTR and β-galactosidase, and the expression levels of these proteins were analyzed by Western blotting. (C, D) Wild-type (WT) (C) and mutant TTR proteins (V30M) (D) purified from human tissues were incubated in vitro with truncated forms of wild-type and C106S DJ-1 [DJ-1 (1-174)] in a buffer containing 0.5 mM sodium acetate (pH 5.5) and 0.2 mM CaCl₂ for 4 h at 37°C. The proteins in the mixtures were analyzed by Western blotting with anti-DJ-1 and anti-TTR antibodies.
H2O2, while DJ-1 in the cells was co-distributed over the range Flag and anti-DJ-1 antibodies (Fig. 5). Without treatment of each fraction were analyzed by Western blotting with anti-

Proteins extracted from cells with an NP-40-containing buffer and proteins in the culture medium were then separated through a 5-20% sucrose density gradient, and proteins in the culture medium were then separated and analyzed by Western blotting with an anti-

Figure 4. Association of DJ-1 with TTR. (A) 293T cells were transfected with an expression vector for Flag-WT-TTR or Flag-V30M-TTR. Forty-eight hours after transfection, proteins in whole cell extracts were immunoprecipitated with an anti-Flag antibody or non-specific IgG, and the immunoprecipitates were then analyzed by Western blotting with an anti-

The results showed that DJ-1 was associated with a tetramer of WT-TTR in 293T cells.

Co-secretion of DJ-1 with TTR. Since TTR secreted from cells forms an amyloid in FAP and the onset of FAP is thought to be related to oxidative stress (37), the distribution of DJ-1 and the conformation of TTR in cells and culture medium were examined. To do this, 293T cells expressing Flag-WT-TTR or Flag-V30M-TTR were established. These cells were treated with 5 μM H2O2 for 20 h or not treated. Proteins extracted from cells with an NP-40-containing buffer and proteins in the culture medium were then separated through a 5-20% sucrose density gradient, and proteins in each fraction were analyzed by Western blotting with anti-

interaction of DJ-1 with TTR at the endogenous level, proteins from human 293T cells were immunoprecipitated with the anti-DJ-1 antibody, and the precipitates were then analyzed by Western blotting with an anti-

Since DJ-1 and TTR have been shown to be secreted into the serum of breast cancer patients (12) and FAP patients (6), respectively, the amounts of DJ-1 in the serum of normal persons, FAP (V30M) patients and V30M-containing carriers were measured by a sandwich ELISA (Fig. 6C). The DJ-1 level in the serum was significantly (p=0.006) higher in FAP (V30M) patients than in normal persons and TTR-V30M-carrier patients, indicating that more DJ-1 was secreted into the serum in FAP patients than in healthy and V30M-containing carriers. Since the oxidative state of DJ-1 determines its biological activity and oxidation of C106 is essential for DJ-1 to exert its full activities, including anti-

Secretion of an inactive form of DJ-1 in the serum of FAP patients. To examine the characteristics of DJ-1 and TTR, proteins from the hearts of FAP patients containing TTR amyloid were separated into 1% Triton X-100-soluble and insoluble fractions and analyzed by Western blotting (Fig. 6A). The majority of TTR and DJ-1 was found to be present in insoluble and soluble fractions, respectively. Localization of TTR and DJ-1 in FAP patients was further examined by an immunohistochemical analysis using hearts and lumbar nerves of FAP patients (Fig. 6B). Congo red staining in the heart sections showed apple-green birefringence characteristic of congophilic amyloid deposits around the interstice of myocardial cells in all the specimens (Fig. 6B-CR), which was confirmed by emission of an emerald-green birefringence in polarized light (Fig. 6B-PL). While all of the amyloid deposits were immunoreactive with an anti-TTR antibody, they were not immunoreactive with an anti-DJ-1 antibody (Fig. 6, TTR and DJ-1). These results indicate that localizations of TTR and DJ-1 in myocardial cells were in a mirror image; TTR was localized in the amyloid and DJ-1 was localized around the amyloid. Results similar to or the same as those in the heart were obtained in the lumbar nerve (Fig. 6B, right part).

Since DJ-1 and TTR have been shown to be secreted into the serum of breast cancer patients (12) and FAP patients (6), respectively, the amounts of DJ-1 in the serum of normal persons, FAP (V30M) patients and V30M-containing carriers were measured by a sandwich ELISA (Fig. 6C). The DJ-1 level in the serum was significantly (p=0.006) higher in FAP (V30M) patients than in normal persons and TTR-V30M-carrier patients, indicating that more DJ-1 was secreted into the serum in FAP patients than in healthy and V30M-containing carriers. Since the oxidative state of DJ-1 determines its biological activity and oxidation of C106 is essential for DJ-1 to exert its full activities, including anti-

Discussion

Structural analysis of DJ-1 has shown that DJ-1 has a structure similar to that of protease I, a cysteine protease of Pyrococcus horikoshii (24), and Oizmann et al have reported that DJ-1 has protease activity using casein as a substrate
Figure 5. Co-secretion of DJ-1 with TTR and abrogation of DJ-1 secretion by oxidative stress. 293(WT-TTR) (A) and 293(V30M) (B) cells were treated with or without 5 μM H₂O₂ for 20 h, and proteins extracted from cells with an NP-40-containing buffer and proteins in the culture medium were then separated through a 5-20% sucrose density gradient. Proteins in each fraction were analyzed by Western blotting with anti-Flag and anti-DJ-1 antibodies.
Figure 6. Immunohistochemical analysis of TTR and DJ-1 in human hearts and lumbar nerves of FAP patients (V30M) and secretion of an inactive form of DJ-1 into the serum of FAP patients. (A) Proteins from hearts of FAP patients (ATTR-V30M) were separated into 1% Triton X-100-soluble (lane 1) and -insoluble (lane 2) fractions and analyzed by Western blotting with anti-DJ-1 and anti-TTR antibodies. (B) Sections were made from human hearts and lumbar nerves of FAP patients (V30M) stained with Congo red and examined under polarized light and were also reacted with anti-DJ-1 and anti-TTR antibodies followed by visualization with HRP-conjugated secondary antibodies. Magnification, x100. CR, Congo red; PL, polarized light. (C) DJ-1 secreted into the serum of FAP patients (V30M) was measured by a sandwich ELISA. p=0.006 compared to normal persons. (D) Proteins in the serum were first immunoprecipitated with the anti-DJ-1 antibody, and then precipitates were analyzed by Western blotting with anti-DJ-1 monoclonal (3E10) and anti-C106 oxidized DJ-1 antibodies.
that degraded in cells transfected with full-sized DJ-1, suggesting NIH3T3 cells, and both wild-type and mutant TTR were substrate for DJ-1 protease. The expression level of TTR in for DJ-1 protease (24, 25). We then identified TTR as a substrate for DJ-1 protease. The expression level of TTR in DJ-1-knockdown D2 cells was higher than that in parental NH3T3 cells, and both wild-type and mutant TTR were degraded in cells transfected with full-sized DJ-1, suggesting that α-helix 9 in DJ-1 was opened or cleaved by DJ-1-binding protein(s) in cells to exert protease activity. Although such DJ-1-binding protein(s) is not identified at present, various chaperones that bind to DJ-1, including Hsp70, Chip and mitochondrial Hsp70, might be candidates (38, 39).

DJ-1 and TTR were co-secreted into the culture medium, in which monomer TTR was degraded and a secretion of DJ-1 was abrogated by oxidative stress, thereby increasing monomer TTR and resulting in the formation of amyloid fibrils. Since the culture medium was first centrifugated at 3,000 x g and its supernatant was used as the culture medium, in which actin was not detected by Western blotting, secreted DJ-1 into the culture medium was not derived from dead cells. It has been shown that a secreted monomer, but not tetramer, of TTR forms amyloid fibrils (3-6). DJ-1 was found to be co-secreted with a tetramer wild-type TTR into the culture medium under normal conditions. Under oxidative conditions, on the other hand, DJ-1 was not secreted and monomer, dimer and tetramer forms of both wild-type and V30M mutant TTR were accumulated in the medium, suggesting that DJ-1 prevents monomer formation of TTR.

The DJ-1 concentration in the serum of FAP patients was significantly higher than that in the serum of healthy persons and V30M-containing carriers. Secreted DJ-1 in the serum of FAP patients was found to be unoxidized DJ-1, which is an inactive form of DJ-1 as to its chaperone activity (27). Since it has not been shown that unoxidized DJ-1 is also the inactive form for DJ-1 protease, we are now examining this possibility. Furthermore, localization and solubility of TTR and DJ-1 in tissues of FAP patients were a mirror image; TTR and DJ-1 were located inside and outside amyloid fibrils, and TTR and DJ-1 were insoluble and soluble. These results suggest that oxidative stress acting on cells abrogates secretion of DJ-1 and that secreted DJ-1 degrades aggregated TTR to protect the onset of FAP. When secreted DJ-1 is inactivated by reduction, monomer TTR aggregates, resulting in the formation of amyloid fibrils.

A mirror image of localization of DJ-1 and α-synuclein in Lewy bodies in tissues of Parkinson’s disease patients has also been reported (40). Furthermore, it has been reported that DJ-1 was secreted into the cerebrospinal fluid of patients with stroke and that DJ-1 may be used as a plasma marker for the early diagnosis of stroke (41). These findings together with our present study suggest that in addition to Parkinson’s disease, DJ-1 is related to oxidative stress-related neurodegenerative disorders.

Acknowledgements

We thank Yoko Misawa and Kiyomi Takaya for their technical assistance. This study was supported by grants-in-aid from the Ministry of Education, Science, Culture, Sports and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan.

References


