DNA adducts in mammalian cells exposed to N-acetoxy-2-acetylaminofluorene* (NA-AAF), an activated derivative of the potent carcinogen 2-acetylaminofluorene (2-AAF), play significant roles in cell killing, chromosome aberration, sister-chromatid exchange, gene mutation and neoplastic transformation (1,2). NA-AAF binds covalently to guanine in the DNA of mammalian cells and produces three different DNA adducts. The C-8 adducts, N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) and deacetylated N-(deoxyguanosin-8-yl)- 2-aminofluorene (dG-C8-AF), account for the major portion of the DNA-bound products, while the minor N2 adduct, 3-(deoxyguanosin-N2-yl)-2-acetylaminofluorene (dG-N2-AAF), accounts for the rest of them. The relative induction levels of the two major C-8 adducts vary among cell types. These adducts distort the DNA helix as do UV-induced cyclobutane pyrimidine dimers (CPD), and therefore they are repaired by nucleotide excision repair in human cells. Iwamoto et al (3) have established monoclonal antibodies against dG-C8-AAF in denatured DNA. These antibodies enable one to detect dG-C8-AAFs in DNA from cultured cells using an enzyme-linked immunosorbent assay (ELISA) and to visualize them in cultured cells or rodent tissues using an immunofluorescence (IF). This technology would contribute to understanding of molecular mechanisms in AAF-related research fields including cancer research, antitumor research or toxicology.

Background

DNA adducts in mammalian cells exposed to N-acetoxy-2-acetylaminofluorene* (NA-AAF), an activated derivative of the potent carcinogen 2-acetylaminofluorene (2-AAF), play significant roles in cell killing, chromosome aberration, sister-chromatid exchange, gene mutation and neoplastic transformation (1,2). NA-AAF binds covalently to guanine in the DNA of mammalian cells and produces three different DNA adducts. The C-8 adducts, N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) and deacetylated N-(deoxyguanosin-8-yl)- 2-aminofluorene (dG-C8-AF), account for the major portion of the DNA-bound products, while the minor N2 adduct, 3-(deoxyguanosin-N2-yl)-2-acetylaminofluorene (dG-N2-AAF), accounts for the rest of them. The relative induction levels of the two major C-8 adducts vary among cell types. These adducts distort the DNA helix as do UV-induced cyclobutane pyrimidine dimers (CPD), and therefore they are repaired by nucleotide excision repair in human cells. Iwamoto et al (3) have established monoclonal antibodies against dG-C8-AAF in denatured DNA. These antibodies enable one to detect dG-C8-AAFs in DNA from cultured cells using an enzyme-linked immunosorbent assay (ELISA) and to visualize them in cultured cells or rodent tissues using an immunofluorescence (IF). This technology would contribute to understanding of molecular mechanisms in AAF-related research fields including cancer research, antitumor research or toxicology.

Product type Primary antibodies
Host Mouse
Source The hybridoma was established by fusion of mouse myeloma cells with Balb/c mouse splenocytes immunized with NA-AAF-modified single-stranded DNA conjugated with methylated BSA. This hybridoma (clone AAF-1) culture supernatant was collected and precipitated with ammonium sulfate. After centrifugation, the pellet dissolved in small volume of double-distilled water was dialysed against PBS. The dialysate was then lyophilized.
Form This antibody is lyophilized form. Reconstitute with 100 µl of distilled water. No preservative is contained.
Volume 100 uL(Lyophilized Powder)
Specificity AAF-1 primarily binds to dG-C8-AAF in denatured DNA, although dG-C8-AF in DNA is also recognized with slightly less efficiency.
Clone AAF-1
Isotype IgG1 (kappa)
Applications Immunocytochemistry: 1/25
ELISA: 1/100
Optimal dilutions should be determined by the end user.
Storage Lyophilized form (Before reconstitution) : store at -20°C.
Reconstituted form : store at -20°C.
After reconstitution, it is stable for at least 1 year when stored at -20°C. It should be divided into small quantity to avoid freezing and thawing many times.
References

For research use only. Not for clinical diagnosis.
Application results

The dose-dependent formation of NA-AAF-induced DNA adducts in human cells. Cells were exposed to NA-AAF for 0.5 h and the formation of DNA adducts in denatured DNA (500 ng/well) was determined using a sensitive-direct-binding ELISA with AAF-1 (1/100). (Details are shown in Ref. 3.)

The formation of NA-AAF-induced DNA adducts in human cells. Cells were exposed to 200 µM NA-AAF or solvent for 0.5 h. After permeabilization and fixation, DNA adducts were visualized by sequential treatment of AAF-1 (1/25) and Alexa Fluor 488 goat anti-mouse IgG conjugate. Nuclear DNA was counterstained with DAPI. (Details are shown in Ref. 3.)
PROTOCOLS:

ELISA

A. The coating of microtiter plates by protamine sulfate

1) Prepare 0.003% protamine sulfate solution in distilled water and stir for 1 hour.
2) Distribute 50 μL / well of the solution to 96 well microtiter plates (Polyvinylchloride flat-bottom, Thermo, Cat. No. 2801, Milford, MA).
3) Incubate the plates at 37°C overnight and coat protamine sulfate on plates by drying completely.
4) Wash the plates three times with 100 μL / well of distilled water.
5) These plates can be stored for long times in dark.

B. Cell culture and NA-AAF exposure

6) Plate cells in 10-cm dishes and culture one or two days.
7) Prepare 100 mM NA-AAF in DMSO and expose cells to NA-AAF (for example ; 0, 25, 50, 100, 150 μM) for 30 minutes at 37 °C.
8) Wash cells twice by 10 mL of Dulbecco’s PBS (DPBS), and then cells were harvested by a cell scraper from the dishes and centrifuged at 10,000 x g for 15 seconds at 4 °C.
9) Cell pellets were stored at –80 °C until processing.

C. DNA isolation

10) Genomic DNA was purified using a QIAamp Blood Kit (QIAGEN, Cat. No. 51104 or 51106).
DNA concentrations were calculated from the absorbance at 260 nm.

D. DNA sample coating to the microtiter plates precoated with protamine sulfate

11) Prepare sample DNA solutions in PBS at the concentration of 10 μg / mL
12) To denature DNA, heat DNA solutions in a hot plate at 100 °C for 10 minutes and chill rapidly in an ice bath for 15 minutes.
13) Distribute 50 μL / well of each denatured DNA solution to protamine sulfate precoated 96 well microtiter plates (use 4 wells for each sample) and dry completely overnight at 37 °C.

E. DNA adduct detection

14) Wash the DNA-coated plates 5 times with 150 μL/ well of PBS-T (0.05% Tween-20 in PBS).
15) Distribute 150 μL well of 2% FBS in PBS to each well to prevent non-specific antibody binding.
16) Incubate 30 minutes at 37 °C.
17) Wash the plates 5 times with 150 μL/ well of PBS-T.
18) Distribute 100 μL / well of AAF-1 antibodies diluted with PBS as suggested in the APPLICATIONS to each well and incubate 30 minutes at 37 °C.
19) Wash the plates 5 times with 150 μL / well of PBS-T.
20) Distribute 100 μL / well of 1:2000 Biotin-F(ab')2 fragment of anti-mouse IgG (H+L) (Rockland, Code 710-106-019) diluted with PBS to each well and incubate 30 minutes at 37 °C.
21) Wash the plates 5 times with 150 μL / well of PBS-T.
22) Distribute 100 μL / well of 1:10000 Peroxidase-Streptavidin (Thermo Fisher Scientific, Cat. No. 43-4323) diluted with PBS to each well and incubate 30 minutes at 37 °C.
23) Wash the plates 5 times with 150 μL/well of PBS-T.
24) Wash the plates once with 150 μL/well of Citrate-phosphate buffer (pH5.0) [Citric acid monohydrate 5.10 g, Na₂HPO₄ 7.30 g, Distilled water 1000 ml]. Keep the buffer solution in the plates until the next substrate solution is ready.
25) After throwing the buffer away, distribute 100 μL/well of the substrate solution [o-Phenylenediame 8 mg, H₂O₂ (35%) 4 μl, Citrate-phosphate buffer (pH5.0) 20 ml] to each well and incubate 30 minutes at 37 °C.
26) Distribute 50 μL/well of 2M H₂SO₄ to each well and stop enzyme reaction.
27) After gentle mixing, determine the absorbance at 492 nm of each well by a spectrophotometer.

**Immunofluorescence microscopy**

**A. Cell culture and NA-AAF exposure**
1) Culture the cells in the appropriate condition in 35-mm glass-bottom dishes (MatTek, Ashland, MA). (For example, inoculate 4x10⁵ cells per dish, then incubate for one or two days in a CO₂ incubator.)
2) Prepare 100 mM NA-AAF in DMSO and expose cells to NA-AAF (for example ; 0, 200 μM) for 30 minutes at 37 °C. Wash cells 2 times with 2 mL of DPBS.

**B. Cell fixation and permeabilization**
3) Pour 1 mL of 0.5% Triton X-100 in PBS, and permeabilize the cells for 15 minutes on ice.
4) Wash the cells 2 times with 2 mL of DPBS.
5) Pour 2 mL of methanol-aceton (1:1) into each dish, and fix the cells for 10 minutes at -20 °C.
6) Dry the dishes at room temperature for 5-10 minutes.

**C. Indirect Immunofluorescence**
7) Add 70 μL of 2M HCl and denature cellular DNA for 30 minutes at room temperature.
8) Wash the cells 5 times with 2 mL of PBS.
9) Add 70 μL of 20% FBS in PBS to prevent non-specific antibody binding.
10) Incubate 30 minutes at 37 °C with gentle shaking.
11) Wash the cells 5 times with 2 mL of PBS.
12) Add 70 μL of AAF-1 antibodies diluted with PBS containing 5% FBS as suggested in the APPLICATIONS onto the cells and incubate for 30 minutes at 37 °C with shaking (Optimization of antibody concentration or incubation condition is recommended if necessary.)
13) Wash the cells 5 times with 2 mL of PBS. (Subsequent steps must be done in the dark.)
14) Add 70 μL of 1:100 Alexa Fluor 488-F(ab')₂ fragment of anti-mouse IgG (H+L) (Molecular Probes, Cat. No. A-11017) diluted with PBS containing 5% FBS and incubate for 30 minutes at 37 °C with shaking.
15) Wash the cells 5 times with 2 mL of PBS.
16) Add 70 μL of 0.05 μg/mL DAPI in PBS and incubate for 5 minutes at 37 °C with shaking.
17) Wash the cells 5 times with 2 mL of PBS.
18) Promptly add 20 μL of Vectashield mounting medium (Vector, Cat. No. H-1000) onto the cells, then put a cover slip on them.
19) Observe fluorescence images by a fluorescence microscope.