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Novel Protein Transfection of Primary Rat Cortical Neurons Using an Antibody That Penetrates Living Cells\textsuperscript{1}

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An Ab-based system to deliver functional proteins into neurons was developed using the murine mAb, mAb 3E10. This was achieved by covalently conjugating catalase to the Ab so that the conjugate retained high activity for the degradation of hydrogen peroxide. Three-dimensional fluorescence microscopy was used to demonstrate penetration of the Ab into the nucleus of living primary cortical neurons. The Ab conjugate localized in both the cytoplasm and nucleus. Retention of catalase activity after penetration and distribution of conjugate was demonstrated by reduction in cell death following exposure of treated neurons to hydrogen peroxide. These studies illustrate the potential of this method for the intracellular delivery of therapeutic proteins. 

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Penetration of living cells by certain Abs has been recognized for many years as recently reviewed (1). A subset of antinuclear Abs can penetrate living cells (2–7), and some can even localize in the nucleus. Some of these Abs produce pathological effects and are of interest for their role in producing symptoms of autoimmune disease (8–13). Other Abs penetrate cells without obvious harmful effects and are of interest for development as therapeutic delivery systems. We recently described a monoclonal anti-dsDNA Ab, mAb 3E10, that penetrates living cells and localizes in the cell nucleus without apparent injury to target cells (14, 15). In this study we explored the development of mAb 3E10 as a delivery system for functional proteins.

Oxidative injury to the nervous system is proposed as a cause of primary neuronal death in hypoxia-ischemia and neurodegenerative diseases such as Alzheimer’s disease (16–18). Hydrogen peroxide is known to cause oxidative injury and death in neuronal cultures (19–23). Furthermore, intracellular increases in antioxidants can protect neurons from oxidative-induced injury (20, 21). Therefore, we tested whether catalase delivered by covalent linkage to mAb 3E10 would protect primary rat cortical neurons from hydrogen peroxide-induced cell death.

Materials and Methods

Monoclonal Ab

mAb 3E10 (IgG2a) was derived from the fusion of spleen cells from an MRL[primpj mouse with FOX-NY hybridoma cells as previously described (24). mAb 3E10 was shown to bind single- and double-strand DNA as well as to penetrate living cells and localize in the cell nucleus (14, 15, 24). mAb 3E10 was purified from hybridoma supernatant by affinity binding to protein A-Sepharose followed by washing with 0.5 M NaCl and then eluting the Ab with Gentle elution buffer (Pierce, Rockford, IL). The purity of Ab was determined by electrophoresis in 12% polyacrylamide gels in the presence of SDS.

FITC conjugates

Complexes containing purified mAb 3E10 (100 μg/ml) and FITC-labeled goat anti-mouse Abs (Southern Biotechnology Associates, Birmingham, AL) were formed in a 1/4 molar ratio. We previously showed that mAb 3E10 penetrated cells and localized in the cell nucleus complexed with alkaline phosphatase-conjugated goat anti-mouse Abs (15).

Live/dead cell assay

Living cells were recognized either by the uptake of the esterase substrate carboxy-2’,7’-dichlorofluorescein diacetate (Molecular Probes, Eugene, OR) or the exclusion of ethidium homodimer (Molecular Probes) or propidium iodide (Sigma, St. Louis, MO). Ethidium homodimer and propidium iodide are excluded from cells with intact cell membranes, but they penetrate dead cells and intercalate into nuclear DNA. Dead cells were identified by adding ethidium homodimer or propidium iodide (8 nM final concentration) to the cells in tissue culture 10 min before microscopic examination.

Colocalization studies

Hoechst 33342 (Molecular Probes) penetrates living cells and binds DNA in the cell nucleus of live or dead cells. This reagent was used to identify cell nuclei in living cells and for colocalization studies to determine whether mAb 3E10 colocalized with DNA in the nucleus. Hoechst 33342 (5 μM final concentration) was added to the cells in tissue culture 10 min before microscopic examination.

Primary culture of rat cortical neurons

Cultures of rat cortical neurons were prepared from cerebral hemispheres of 16-day-old fetal Wistar rats (25). Hemispheres were dissected under sterile conditions and mechanically dissociated and plated in polylysine-coated 30-mm round coverslips placed in six-well plastic dishes (Corning Costar, Cambridge, MA). Cells were cultured for 7–10 days before the experiments. The defined feeding medium was previously described (25).

Cellular penetration

Perfusion chambers (C Perfusion chambers, Research Products International, Mount Prospect, IL) were mounted onto 35 × 50-mm cover glasses, sterilized by autoclaving, and then seeded with 3 × 10\(^{5}\) COS-7 cells. The cells were grown to confluence in 3 days after culturing them at 37°C in DMEM with 10% heat-inactivated FCS. Complexes of mAb 3E10 and FITC-conjugated goat anti-mouse Abs were then incubated with the cells for 10 min and imaged by fluorescence three-dimensional microscopy.
Protection of primary neurons from injury by hydrogen peroxide

Primary rat cortical neurons were cultured in normal feeding medium with or without added hydrogen peroxide (300 μM) for 24 h and examined for viability by the exclusion of propidium iodide or the presence of esterase activity. Before the addition of hydrogen peroxide, selected neurons were incubated with free catalase (300 μg/ml), free mAb 3E10 (20 or 40 μg/ml), and mAb 3E10-catalase conjugate (equivalent to 20 or 40 μg/ml of Ab) for 1 h, washed three times in DMEM/F-12 medium without serum, and incubated with hydrogen peroxide. The catalase activity was measured by the method of Beers and Sizer (27). The amount of free active catalase used as a control was calculated to be greater than 2 times the amount of catalase activity present in the conjugate based on an amount of conjugate equivalent to 40 μg/ml of Ab. The cells were assayed for viability and then fixed with methanol. The fixed cells were evaluated for localization of mAb 3E10 with alkaline phosphatase-conjugated goat Abs reactive with mAb 3E10 with alkaline phosphatase-conjugated chicken Abs specific for goat Abs. Alkaline phosphatase was detected with the substrate, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The assays were performed in triplicate and repeated three times.

Ab-catalase conjugate

mAb 3E10 (10 mg) was conjugated covalently to bovine catalase (Worthington, Freehold, NJ) in a 1:1 molar ratio with the use of two different heterobifunctional reagents, succinimidyl 3-(2-pyridyldithio)propionate and succinimidyl trans-4-maleimidylmethyl)cyclo-hexane-1-carboxylate, to modify the lysine residues of one protein to thiols and to add thiol-reactive maleimide groups to the other protein. After deprotection of the thiolated protein by tris-(2-carboxyethyl)phosphine, the two modified proteins were reacted to each other, forming a stable thio-ether bond between them. The conjugation was performed at Molecular Probes and was fractionated by gel filtration chromatography. The composition of the fractions was assessed in our laboratory by electrophoresis of the fractions in native and SDS-polyacrylamide gels in reducing and nonreducing environments. Pooled fractions containing 98% conjugate, <1.0% free catalase, and <2% free Ab were selected for use in our studies. Catalase activity of the conjugate was assayed at 40% of catalase enzyme alone on a milligram per milligram comparison (27).

False color images and three-dimensional microscopic imaging with false color colocalization and stereo pair formation

An inverted Olympus IX-70 microscope (New Hyde Park, NY) using epifluorescent illumination was used to collect all images. Conventional images were captured with a 12-bit cooled CCD camera (Sensys, Photometrics, Tucson, AZ) and false colored according to the filter cube set used. For ethidium homodimer (red fluorescence) a standard rhodamine filter cube was used, and gray intensities from the camera were replaced with equal intensities of red. The green (26) and blue (Hoechst 33342) fluorescence images were obtained with a fluorescein filter cube and a fura-2 filter cube (380 nm excitation >500 nm emission), respectively, followed by replacement of gray intensities by equal intensities of the appropriate color.

Optical sections were obtained with the above microscope equipped with a ×100, 1.35NA oil immersion lens. Widefield images were collected under control of CELLscan System software from Scanalytics (Billerica, MA), which controlled a piezoelectric z-axis focus device and computer-controlled shutter. The resulting image stack was deblurred using Scanalytics Exhaustive Photon Reassignment software run on a Sun/Microsparc workstation (Mountain View, CA).

For colocalization studies, monochrome deblurred images collected with the blue and green fluorescence cubed were background subtracted then combined using the binary and to produce a stack of image masks where blue and green fluorescence was colocalized. These colocalization masks were then used with the original image stacks to produce noncolocalization masks for the blue and green fluorescence image sets. To present the colocalization data, the colocalization mask (colored red) and the blue and green fluorescence noncolocalization masks were merged as RGB color planes.

Stereo pairs were prepared using CELLscan System software that allows rotation of the image stacks (optical sections). Left and right stereo images were obtained by slight (3° to 5°) rotations left and right.

Results

Monoclonal Ab 3E10 penetrated living COS-7 cells and localized in the nucleus

We first studied penetration of mAb 3E10 and mAb 3E10 conjugated to catalase in COS-7 cells, because these cells were used as a model system in previous studies. In our earlier studies, however, Ab was visualized after penetration and fixation of cells (14, 15). In the current studies we examined Ab penetration while cells were alive to avoid aberrant localization attributable to artifacts of cell fixation. This was done by using FITC-labeled mAb 3E10 to COS-7 cells in the presence of Hoechst 33342 and ethidium homodimer. Hoechst 33342 was visualized in living and dead cells as a blue color in the cell nuclei, as shown in Fig. 1A. The ethidium homodimer identified only dead cells as those with red nuclei (Fig. 1C). The nuclei containing FITC-conjugated Ab (green) are shown in Fig. 1B. All cell nuclei contained Ab, as shown by comparison with the Hoechst dye. This was expected, because mAb 3E10 localized to the nuclei of both live and dead cells. If mAb 3E10 bound only the DNA of dead cells, this pattern would not be expected. However, Fig. 1B clearly shows that mAb 3E10 penetrated living cells (negative stain for ethidium homodimer) with intact cellular membranes and localized to the nucleus. Multiple images were recorded at 0.5-μm intervals through the nucleus to determine whether the Ab was distributed on the outside of the nuclear membrane or whether it had penetrated the intranuclear compartment. Each of the sections showed the presence of Ab distributed throughout the entire nucleus. A composite was made of multiple images throughout the nucleus, and a stereo image was created by rotation of the composite image (Fig. 1D). The stereo image demonstrated that the Ab was inside and distributed throughout the cell nucleus.

Colocalization of mAb 3E10 and DNA in living COS-7 cells

Studies were performed with Hoechst 33342 that binds DNA to determine whether mAb 3E10 colocalized with DNA in the nucleus. Living cells were identified by the absence of staining with ethidium homodimer. Colocalization of the Ab and DNA was assessed in composite images of multiple cells that did not stain with ethidium homodimer. A typical example is shown in Fig. 2. The distribution of FITC-labeled Ab (green) is shown in Fig. 2A. Hoechst 33342 (blue) showed the distribution of DNA in the nucleus (Fig. 2B). These images were superimposed, and the overlap of Ab (green) and DNA (blue) was indicated by a red color (Fig. 2C). The red color in this figure does not represent ethidium homodimer and should not be confused with ethidium homodimer in Fig. 1C. The cell shown in Fig. 2 did not stain with ethidium homodimer at the beginning or at the conclusion of the experiment, indicating that the cell was alive throughout the period of observation. The red area in Fig. 2C indicated colocalization of mAb 3E10 and DNA. Thus, the Ab localized with DNA in the nucleus.

Monoclonal Ab 3E10 penetrated living primary rat cortical neurons and localized in the nucleus

Although mAb 3E10 can penetrate dividing cells such as COS-7 cells, we wanted to determine whether the Ab could also penetrate nondividing cells. We used primary rat cortical neurons as a source of nondividing cells. The cortical neurons were grown in cell culture, as shown in the light photomicrograph (Fig. 3D). The cell nuclei were identified by Hoechst 33342 (Fig. 3A), and the dead cells were distinguished from the living cells by ethidium homodimer (Fig. 3C). As shown in Fig. 3B, the FITC-labeled Ab was visualized in the living cells.
penetrated living primary cortical neurons and localized in the cell nucleus.

To establish that the second Ab (goat anti-mouse) alone did not penetrate living cells and localize in the nucleus, we studied theFITC-conjugated goat anti-mouse Ab in the absence of mAb 3E10-penetrating Ab. The cell nuclei of primary cortical neurons were identified by Hoechst 33342 (blue; Fig. 3E), and the dead cells (red) were distinguished from the living cells by ethidium homodimer (Fig. 3G). The FITC-conjugated goat anti-mouse Ab alone showed nonspecific binding to the nuclei of dead cells, but it did not penetrate living cells (Fig. 3F). The same result was demonstrated with the FITC-conjugated goat Ab and COS-7 cells (data not shown).

Colocalization of mAb 3E10 and DNA in living, primary rat cortical neurons

Colocalization of the Ab and DNA was assessed in primary cortical neurons by the same method as that used in COS-7 cells. Cells that did not stain with ethidium homodimer were examined for the distribution of Ab and DNA. The distribution of Ab (green) in a representative living primary rat cortical neuron is shown in Fig. 4A, and the distribution of Hoechst 33342 (blue) is shown in Fig. 4B. Areas in these figures that contain both Hoechst (blue) and Ab (green) have been colored red, indicating colocalization (Fig. 4C). As shown in Fig. 4C, the chromatin is more tightly packed in primary neurons than in COS-7 cells.

Monoclonal Ab 3E10-catalase conjugate penetrated COS-7 cells

We next produced conjugates of mAb 3E10 and catalase to determine whether mAb 3E10 could transport catalase into COS-7 cells and primary cortical neurons. COS-7 cells were incubated with mAb 3E10 alone (Fig. 5, A and D), catalase alone (Fig. 5, B and E), and mAb 3E10-catalase conjugate (Fig. 5, C and F). The cells were fixed and examined for the presence of mAb 3E10 with alkaline phosphatase-conjugated Abs reactive with mouse κ-chains (Fig. 5, A–C) and for the presence of catalase with alkaline phosphatase-conjugated Abs reactive with bovine catalase (Fig. 5, D–F). mAb 3E10 penetrated COS-7 cells and localized in the nucleus (Fig. 5A), whereas mAb 3E10-catalase conjugate localized in both the cytoplasm and nucleus (Fig. 5C). Staining for the presence of catalase showed the presence of endogenous catalase in COS-7 cells (Fig. 5D). However, incubating the cells with free catalase followed by washing did not increase the amount of intracellular catalase (Fig. 5E). In addition, there was no evidence of the free catalase bound to the plasma membranes of COS-7 cells. However, there was a marked increase in cytoplasmic and nuclear catalase in cells incubated with the Ab-catalase conjugate, as identified with alkaline-phosphatase conjugated Abs directed to catalase (Fig. 5F). These results suggest that intact covalently linked Ab-catalase complexes penetrated COS-7 cells and localized in the cytoplasm and nucleus. The viability of the COS-7 cells at the...
The conclusion of the experiments was >90%, as shown by the exclusion of trypan blue. Therefore, the Ab-catalase complexes were demonstrated to penetrate living cells.

Monoclonal Ab 3E10-catalase conjugate penetrated living rat primary cortical neurons and protected the cells from injury by hydrogen peroxide

We next assessed mAb 3E10-catalase conjugate for penetration into primary rat cortical neurons and protection of the neurons from injury in the presence of hydrogen peroxide. Neurons were incubated in the absence (Fig. 6, A–C) and the presence (Fig. 6, D–F) of hydrogen peroxide after pretreatment with catalase alone (Fig. 6, A and D), mAb 3E10 alone (Fig. 6, B and E), and Ab-catalase conjugate (Fig. 6, C and F). After performing a live/dead assay, the cells were stained for the presence of mAb 3E10, and the integrity of the neurons was assessed by morphological appearance. mAb 3E10 alone penetrated neurons and localized in the nucleus (Fig. 6B), whereas mAb 3E10-catalase conjugate localized in the cytoplasm and nucleus (Fig. 6C) similar to the results obtained in COS-7 cells. Whereas catalase alone (Fig. 6D) and Ab alone (Fig. 6E) did not protect the neurons from oxidative injury, the conjugate protected neurons in the presence of hydrogen peroxide (Fig. 6F). The conjugate was not observed in all the neurons. However, the neurons containing the conjugate showed preferential survival compared with those without conjugate. The live/dead cell assay (Fig. 7) confirmed the protection of living neurons from oxidative cell death in response to hydrogen peroxide. In three separate experiments, the viability of neurons incubated with 20 μg/ml of mAb 3E10-catalase was 40–50%, and the viability with 40 μg/ml of mAb 3E10-catalase was 60–70%. The results of a representative experiment are shown in Table I. All experiments were performed in triplicate, and consistent results were obtained in each experiment.

Discussion

In this report we definitively demonstrated Ab penetration and localization into the nucleus of living cells. In our previous work we demonstrated Ab penetration into dividing cells, and the Ab was localized in cells fixed after penetration. Cell fixation may produce artifacts of localization, and disruption of the nuclear membrane may permit the entrance of Ab into the nucleus of dividing cells. To overcome these technical limitations, we developed a method to identify penetrating Ab inside of living, nondividing cells.

We showed for the first time that mAb 3E10 localized to the nucleus of living cells before cell fixation using three-dimensional fluorescence imaging. Moreover, we demonstrated colocalization
of mAb 3E10 and DNA in the nucleus. The influence of cell division on Ab penetration was excluded by studying nondividing primary rat cortical neurons. The penetration of living cells by mAb 3E10 complexed with an FITC-conjugated goat anti-mouse Ab confirmed earlier findings that mAb 3E10 can serve as a delivery system to transport large protein complexes into the cell nucleus. To extend our previous work we concentrated on delivering a functional cellular protein to protect cells from oxidative stress.

Oxidative-induced cell death is important in several human diseases, including cardiovascular disease (28) and neurological disorders such as Alzheimer’s disease and stroke (16–18). The intracellular mechanisms for protection against oxidative stress include antioxidant free radical scavengers, such as vitamin E and C, and antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, and catalase. Hydrogen peroxide is produced from superoxide anions and hydrogen ions by the antioxidant enzyme superoxide dismutase and degraded to water by catalase or glutathione peroxidase (18). If not degraded, hydrogen peroxide can react with ferrous iron to produce reactive oxygen species such as hydroxyl radicals. These are very highly reactive with proteins, lipids, and DNA. The brain is relatively deficient in the antioxidant enzymes (29, 30), so that even mild or intermittent oxidative injury may cause neuronal damage or death. In previous studies it was shown that intracellular increases in antioxidants protected neurons from oxidative-induced injury (20, 21). Delivery of intracellular antioxidants and antioxidant enzymes would presumably protect neurons; however, the delivery of enzymes is impeded by the inability of these enzymes to cross the cellular membrane.

Our goal was to develop a penetrating Ab as an intracellular delivery system to protect neurons from oxidative injury. In previous work we identified an anti-DNA Ab, mAb 3E10, that penetrated living cells and localized in the cell nucleus without apparent harm to the cells (14). The Ab was shown to transport an alkaline phosphatase-conjugated goat anti-mouse Ab (305-kDa complex) to the cell nucleus where the enzyme retained its biological activity, as demonstrated by the conversion of its chromogenic substrate applied after cell fixation (15). This model system demonstrated the feasibility of intracellular delivery of an enzyme, although it did not illustrate a useful biological application.

In the present study we conjugated the antioxidant enzyme catalase (250 kDa) to mAb 3E10 and examined its ability to penetrate living cells and protect them from oxidative stress. An in vitro model for neuronal cell death in neurodegenerative diseases and stroke has been developed in which primary cortical neurons undergo oxidative-induced injury after exposure to hydrogen peroxide (19–23). We used this model to determine whether the Ab-catalase conjugate could protect neurons from oxidative cell death.
induced by hydrogen peroxide. We examined COS-7 cells and neurons for penetration by the Ab-catalase complex. As shown by labeled Abs reactive with mouse Ig and catalase, the Ab-catalase complex penetrated cytoplasmic membranes and localized in both the nucleus and the cytoplasm. The altered distribution of the complex compared with that using Ab alone could be due to many factors, including size, structure, charge, and binding to intracellular sites.

Ab alone did not protect neurons from oxidative injury from hydrogen peroxide. Moreover, catalase alone with an activity greater than 2 times that estimated to be present in the conjugate resulted in a marked increase in intracellular catalase compared with the amount of endogenous catalase. Therefore, the single-chain Ab of mAb 3E10 penetrates cells comparable to the whole Ab (15). Therefore, the single chain mutant Ab may be a more efficient vehicle for the delivery of catalase into neurons and may enhance the feasibility of its use in vivo. Further studies are needed to address the critical issues related to targeting, transport, and protection of neurons in vivo.

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References


