Microtiter Immunocytochemical ELISA Assay

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Quantitative assessment of immunoreactivity and protein expression in cultured cells can be time-consuming and subjective. The problem is magnified when rapid and efficient screening is required, and the supply of cells is limited. Immunoblot analysis requires protein extraction, quantification of protein content and gel electrophoresis, which make the method impractical for evaluating multiple treatment effects or screening for inducible gene expression in a large number of stably transfected clones. Although the use of bioluminescent reporter genes has substantially reduced some of the problems related to clone selection, not all systems can take advantage of such reagents. In this report, we describe the microtiter immunocytochemical enzyme-linked immunosorbent assay (ELISA), called the MICE assay, which is a rapid method for objectively quantifying levels of immunoreactivity in cultured cells without the need for protein extraction, gel electrophoresis or cell counting. The MICE assay differs from the cellular ELISA in that a correction for cell density is incorporated into the procedure, thereby permitting comparisons of protein expression following different treatments, even if the effect of the treatment includes cell death or proliferation. In addition, the MICE assay measures levels of protein expression and intracellular immunoreactivity, whereas cellular ELISAs are designed to detect surface immunoreactivity (2,3,10). The Midwestern assay applies ELISA technology to tissue sections (13), but it provides no means to compare levels of protein expression in relation to cell density. Also, the assay is performed on microscope slides, rendering it difficult to conduct multiple simultaneous analyses.

This report describes two examples in which the MICE assay was used to measure changes in protein expression. One study examined the inhibitory effects of ethanol on insulin-stimulated protein expression, and the other mea-

sured nitric-oxide synthase 3 (NOS3) levels in cells infected with recombinant adenovirus vectors (Adv) that express full-length NOS3 or green fluorescent protein (GFP) cDNA under the control of a cytomegalovirus (CMV) promoter. The objective was to demonstrate that modulation of endogenous genes and genes over-expressed by infection or transfection of foreign cDNAs is readily detected and quantified using the MICE assay.

Insulin stimulation studies. Insulin stimulation modulates cell proliferation and energy metabolism by activating complex intracellular signal transduction pathways (11,15). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an insulin-responsive gene (1), and insulin-stimulated DNA synthesis is also associated with increased levels of proliferating cell nuclear antigen (PCNA) (4). Ethanol has profound inhibitory effects on insulin-stimulated DNA synthesis and gene expression (4,16). These adverse effects of ethanol are partly mediated by inhibition of the insulin-stimulated phosphorylation of molecules that are required for intracellular signaling (12,14). NIH3T3 cells were used to examine the inhibitory effects of ethanol on insulin-stimulated GAPDH and PCNA expression. The cells were grown for 4 days in either the presence or absence of 100 mM ethanol (6), then re-seeded (2×10^5 cells per 10-cm² petri dish or 2×10^4 cells per well of 96-well, flat-bottom plates) and maintained in sealed chambers equilibrated with 5% CO₂: 95% air mixture $\pm$ 8 mg/mL ethanol in the reservoir tray (9). After 6 h, the freshly seeded cells were serum-starved for 24 h, then stimulated with 100 nM insulin for 24 h. GAPDH and PCNA expression were assessed by western blot analysis and the MICE assay.

NOS3 expression in neuronal cells infected with recombinant Adv. SH-Sy5y neuroblastoma cells express very low endogenous levels of NOS3 (5). Therefore, these cells were ideally suited to measure increased levels of NOS3 after infection with recombinant Adv that express a full-length cDNA encoding NOS3 or GFP under control of a CMV promoter (8). This model system was used to demonstrate that increased levels of a gene product expressed after

Benchmarks

introduction of foreign DNA could be measured easily by the MICE assay. SH-Sy5y cells, maintained as described previously (17), were seeded at a density of 2×10^5 cells per 10-cm² petri dish or 2×10^4 cells per well in 96-well microplates. After overnight growth, the cells were infected with 0, 10, 20, 30, 50 or 100 multiplicities of infection (MOI) of Adv-NOS3 or Adv-GFP, and NOS3 expression was examined 48 h later. The cells were also examined by fluorescence microscopy to detect GFP expression.

MICE assay. Unless otherwise indicated, all incubations and treatments were performed using reagent volumes of 100–150 μ L per well, and carried out at room temperature with gentle platform agitation. In addition, except for the blocking step, all incubations were followed by 3 rinses in TBS buffer (10 mM Tris-HCl, pH 7.5, 0.85% wt/vol NaCl). The rinses were performed by submerging the plates in a TBS bath to fill the wells, followed by 2 min of gentle platform agitation and then rapid inversion of the plates to discard the buffer.

After discarding the culture medium by rapidly inverting the plate over a sink, the cells were fixed immediately for 15 min in HistoCHOICE™ Solution (AMRESCO, Solon, Ohio, USA) or 2% paraformaldehyde (pH 7.2). The cells were then permeabilized by a 10-min treatment with 0.05% saponin in TBS. Endogenous peroxidase activity was quenched by a 10-min treatment with 0.03% H₂O₂ in TBS. Nonspecific binding sites were blocked by a 15-min incubation with SuperBlock™ (TBS) Solution (Pierce Chemical, Rockford, IL, USA). The cells were then incubated with primary antibody, either overnight at 4°C or for 1 h at room temperature. Both methods yielded satisfactory results, but overnight incubations were more convenient. The monoclonal antibodies to GAPDH (Chemicon International, Temecula, CA, USA), PCNA (Oncogene Research Products, Cambridge, MA, USA) and NOS3 (Transduction Laboratories, Lexington, KY, USA) were used diluted to 0.5 μ g/mL in TBS containing 0.05% Tween® 20 and 0.5% bovine serum albumin (TBST-BSA). Antibody binding was detected by incubating the cells for 30

min at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Pierce Chemical) diluted 1:10 000 in TBST-BSA. Immunoreactivity was revealed by the addition of 100 μ L per well of TMB Soluble Peroxidase Substrate (Pierce Chemical) that was prepared by mixing equal parts of the substrate and hydrogen peroxide solution immediately before use. The color development was stopped before saturation by adding 100 μ L per well of 1 M H₂SO₄. The absorbances were measured at 450 nm using Vmax™ Kinetic Microplate Reader, Molecular Devices (Sunnyvale, CA, USA). To directly visualize cellular labeling, an insoluble chromogen such as 3-3'-diaminobenzidine (DAB) (0.5 mg/mL) or TrueBlue® Peroxidase Substrate (Kir-

kegaard & Perry Laboratories, Rockville, MD, USA) was reacted with the bound HRP-conjugated antibody. DAB yields a brown precipitate, whereas TrueBlue Peroxidase Substrate produces a bright blue staining reaction.

To accurately assess the levels of protein expression, it was necessary to correct for differences in cell density among the wells. Preparatory studies demonstrated that fixed cells could be uniformly stained with Coomassie® Blue dye (0.05% Coomassie Blue dissolved in 40% methanol/10% acetic acid) and that the absorbance values (650 nm) increased linearly with cell density between 1×10^4 and 5×10^5 cells per well ($r^2 = 0.997$). To assess cell number after measuring immunoreactivity, the cells were rinsed with TBS

Figure 1. Comparison between western blot analysis and MICE assay results using SH-Sy5y cells infected with recombinant Adv that express either NOS3 or GFP under control of a CMV promoter. The cells were analyzed for NOS3 or GFP under control of a CMV promoter. The cells were analyzed for NOS3 expression 48 h after inoculation 0–100 multiplicities of infection (MOI) of Adv-NOS3 or Adv-GFP. Panel A depicts western blot data and detection of the approximately 140 kDa NOS3 protein after infection with MOIs of Adv-NOS3 or Adv-GFP. Panel B depicts the mean $\pm$ SD of the MICE indices corresponding to the levels of NOS3 expression in cells cultured in 96-well microplate and infected with different MOIs of the two recombinant vectors.

Benchmarks

and stained for 10 min with 50 μ L per well of Coomassie Blue dye. The plates were then washed extensively by repeated submersions in a bath of running warm (tepid) tap water until no further dye was eluted. After air-drying, the proteins complexed with dye were solubilized in 200 μ L of 1% sodium dodecyl sulfate (SDS) in TBS. The absorbances were measured at 650 nm using an Vmax microplate reader. The MICE index was calculated from the ratio of the absorbances for immunoreactivity and Coomassie Blue labeling (cell density). The means and standard deviations (SD) obtained for eight replicate culture wells were used in the data analysis.

Effects of ethanol on GAPDH and PCNA expression in NIH3T3 cells.

Western blot analysis performed with cell lysates generated from 10-cm² petri dish cultures (4) demonstrated low levels of GAPDH and PCNA in serum-starved cultures. Within 24 h of insulin stimulation, control cells exhibited 80%–100% increases in GAPDH and 30%–40% increases in PCNA expression. Ethanol treatment was associated with markedly reduced levels of both the serum-starved levels and insulin-stimulated increases in GAPDH (0%–2%) and PCNA (10%–15%) expression (6). Using the MICE assay, 250%–300% increases in GAPDH and 350%–400% increases in PCNA were detected in insulin-stimulated control cultures. Corresponding with the results of western blot analysis, the insulin-stimulated responses detected by the MICE assay were muted in the ethanol-treated cultures. In contrast to the striking responses in control cultures, only 30%–40% increases in GAPDH, and 5%–10% increases in PCNA expression were detected in ethanol-treated, insulin-stimulated cells.

Detection of NOS3 expression in SH-Sy5y cells infected with recombinant Adv expressing full-length NOS3 or GFP cDNA. Western blot analysis demonstrated high-level expression of an approximately 140-kDa molecule corresponding to NOS3 in cells infected with Adv-NOS3 and low endogenous levels of NOS3 in SH-Sy5y cells infected with Adv-GFP (Figure 1). GFP expression in Adv-GFP-infected cells (but not Adv-NOS3-infected cells) was verified by

fluorescence microscopy (data not shown). Using the MICE assay, 10-fold to 20-fold higher levels of NOS3 were detected in cells infected with Adv-NOS3 compared with cells infected with Adv-GFP (Figure 1).

The MICE assay provides information similar to that obtained by immunoblot analysis, but it is more sensitive since very low levels of protein are detectable by this technique. However, before using the MICE assay, it is important to verify the molecular mass of the protein detected and the binding specificity of the primary antibodies. In general, the same concentrations of primary antibody can be used for western blot analysis and the MICE assay. As blocking agents, we also tested 0.5% bovine serum albumin (BSA) and 1%–3% nonfat dry milk prepared in TBS, but found the lowest background to be achieved with SuperBlock-TBS. The results were not affected by the manufacturer source of the 96-well microplates. We did not evaluate the utility of round-bottom or conical-bottom plates. The MICE assay may not be ideally suited for non-adherent cells because multiple centrifugation steps would be required between steps.

Three major advantages of the MICE assay are that (i) it can be performed rapidly, (ii) it enables screening for the effects of a large number of compounds and treatments at different doses and (iii) it can be used to screen for inducible gene expression in a large number of clones. Recently, the MICE assay was used in our laboratory to identify clones that had low-basal- and high-induced-levels of neuronal thread protein expression (7). In those studies, inducible gene expression was effected using the LacSwitch™ II Inducible Mammalian Expression System (Stratagene, La Jolla, CA, USA), and the selection of positive clones was verified by immunoblot analysis. In addition to quantifying changes in gene expression, the assay provides information about cell viability and growth in response to treatment or gene activation. Therefore, the MICE assay can be applied to a wide range of experiments that require labor-intensive screening and quantification of immunoreactivity, particularly for complex studies that include multiple variables.

Benchmarks

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