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Molecules and Cells

Quantitative and Rapid Analysis of Transglutaminase Activity Using Protein Arrays in Mammalian Cells

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We developed a novel on-chip activity assay using protein arrays for quantitative and rapid analysis of transglutaminase activity in mammalian cells. Transglutaminases are a family of Ca^{2+}-dependent enzymes involved in cell regulation as well as human diseases such as neurodegenerative disorders, inflammatory diseases and tumor progression. We fabricated the protein arrays by immobilizing N,N′-dimethylcasein (a substrate) on the amine surface of the arrays. We initiated transamidating reaction on the protein arrays and determined the transglutaminase activity by analyzing the fluorescence intensity of biotinylated casein. The on-chip transglutaminase activity assay was proved to be much more sensitive than the [^{3}H]putrescine-incorporation assay. We successfully applied the on-chip assay to a rapid and quantitative analysis of the transglutaminase activity in all-trans retinoic acid-treated NIH 3T3 and SH-SY5Y cells. In addition, the on-chip transglutaminase activity assay was sufficiently sensitive to determine the transglutaminase activity in eleven mammalian cell lines. Thus, this novel on-chip transglutaminase activity assay was confirmed to be a sensitive and high-throughput approach to investigating the roles of transglutaminase in cellular signaling, and, moreover, it is likely to have a strong potential for monitoring human diseases.

INTRODUCTION

Transglutaminases (TGs) are a family of enzymes that are involved in the Ca^{2+}-dependent post-translational modifications of proteins (Griffin et al., 2002; Ruan and Johnson, 2007). TGs catalyze the formation of an ε-(γ-glutamyl)lysine bond between the γ-carboxamide group of a peptide-bound glutamine and the ε-group of a peptide-bound lysine. A (γ-glutamyl)polyamine bond between a peptide-bound glutamine and a polyamine is also catalyzed by TGs. In addition, TGs catalyze the deamination of proteins, as well as the incorporation of serotonin into small GTPases during activation and aggregation of platelets (Ruan and Johnson, 2007; Sane et al., 2007; Walther et al., 2003).

TGs are involved in various functions, dependent or independent of their transamidating activity, including the formation of cornified cell envelopes, neurodegenerative disorders, inflammatory and degenerative diseases, and tumor progression (Esposito and Caputo, 2005; Kim, 2006; Kotsakis and Griffin, 2007; Sane et al., 2007). It is known that tissue transglutaminase (TG2), the most well-characterized among the TGs, is involved in cataratogenesis, celiac disease and neurodegenerative disorders including Huntington's disease, Alzheimer's disease, Parkinson's diseases, amyotrophic lateral sclerosis and nervous system injuries (Gillet et al., 2007; Reif and Lerner, 2004; Ruan and Johnson, 2007). TG2, additionally, plays important roles in cellular responses such as cytoskeletal reorganization, stabilization of extracellular matrix, cell migration and proliferation, and apoptosis (Esposito and Caputo, 2005; Verderio et al., 2004). Factor XIII is a plasma TG and plays an important role in haemostasis, wound healing and the maintenance of pregnancy (Esposito and Caputo, 2005).

Because of the significant functions of TGs in cell regulation as well as in human diseases, a number of assays have been reported for the determination of TG activity (Jeon et al., 2003; Wu and Tsai, 2006). Polyamine incorporation assay using radioactive isotopes or biotinylated polyamines has been used to determine TG activity in cell and tissue extracts (Jeon et al., 2003). However, this incorporation assay is time-consuming, and, significantly, the predominantly used putrescine-incorporation assay, due to its utilization of radioactive isotopes, is allowed only in special restricted areas (Karpati et al., 2000). Photometric assay was reported to determine TG activity by monitoring photometric changes caused by a direct transamidation reaction or an indirect secondary enzyme reaction (De Macédo et al., 2000; Karpati et al., 2000; Wu and Tsai, 2006). TG activity was also determined by enzyme-linked colorimetric assay, which detects biotinylated TG substrates on 96-well microtiter plates by the procedures similar to enzyme-linked immunosorbent assay (Jeon et al., 2006; Trigwell et al., 2004). In addition, there is a recent report on a separation-free fluorometric assay on 96-well microplates using magnetic dextran-coated charcoal (Wu and

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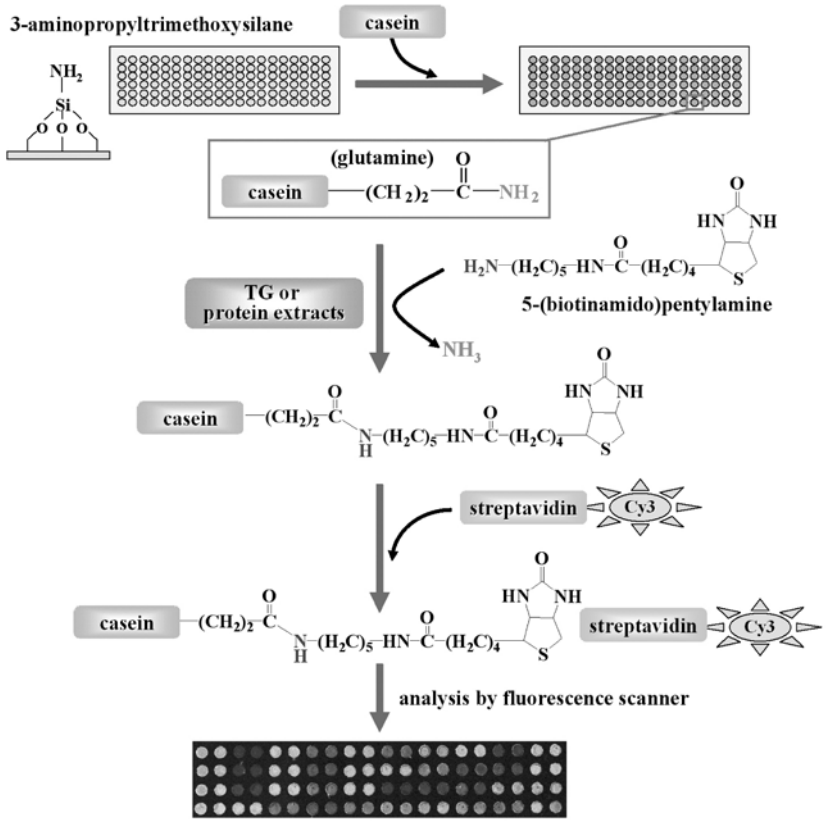

Fig. 1. Schematic diagram of on-chip TG activity assay on protein arrays. Protein arrays were prepared by immobilizing N,N′-dimethylcasein on the amine surfaces of arrays, and the TG activity on the protein arrays was determined as described under "Materials and Methods".

Tsai, 2006). However, there is no report on a protein array-based determination of TG activity in a high throughput manner.

In this paper, we present a novel array-based TG activity assay using protein arrays (an on-chip TG activity assay). In this assay, we fabricated protein arrays by immobilizing N,N′-dimethyl casein on the amine surfaces of arrays, and determined TG activity by analyzing the arrays with a fluorescence scanner, as summarized in Fig. 1. This on-chip activity assay is simple, quantitative, highly sensitive, and it was performed in a very small sample and high-throughput format. Thus, this novel approach has a strong potential for monitoring TG-related human diseases and investigating roles of TG in cellular signaling.

MATERIALS AND METHODS

Chemicals and reagents

3-Aminopropyltrimethoxysilane, ammonium hydroxide, hydrogen peroxide, bovine serum albumin, Cy3-conjugated streptavidin and N,N′-dimethyl casein were obtained from Sigma (USA). Monoclonal anti-TG2 antibody (CUB7402) was purchased from NeoMarker (USA). Fetal bovine serum, penicillin/streptomycin solution, DMEM and RPMI 1640 medium were acquired from Gibco-BRL (USA). 5-(Biotinamido) pentylamine and [1,4(n)-^{3}H]putrescine dihydrochloride were obtained from Pierce Science (USA) and Amersham Bioscience (Sweden), respectively. TG from guinea pig liver was purchased from Oriental Yeast (Japan).

Cell culture

AGS, B16F10, BEAS-2B, HeLa, HCT116, J82 and Raw cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under humidified 5% CO_2. Swiss3T3, NIH 3T3 and SH-SY5Y cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. To induce TG2 expression, NIH 3T3, SH-SY5Y cells were treated with 10 μM all-trans retinoic acid (RA) in the culture medium for 6 days, and the medium was changed every two days. Human umbilical vein endothelial cells (HUVECs) were isolated and grown as described previously (Yi et al., 2006). Cell extracts were prepared by cell sonication in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, protease inhibitor cocktail) followed by centrifugation at 13,000 rpm for 10 min.

Immunoblotting

Cell extracts were resolved by 10% SDS-polyacylamide gel electrophoresis and transferred into a nitrocellulose membrane with a wet-transferring system (Bio-Rad Laboratories, USA). Following incubation with a blocking solution containing 5% skim milk, 0.1% Tween 20, 150 mM NaCl and 20 mM Tris (pH 7.6), the membrane was probed with monoclonal anti-mouse TG2 antibody (1:500, v/v) or anti-mouse β-actin antibody (1:2,000, v/v), and a horseradish peroxidase-conjugated anti-mouse IgG (1:2000, v/v). The protein bands of TG2 and β-actin were then detected by the ECL™ system (Pierce Science, USA). The intensity of TG2 bands, analyzed by densitometry, was normalized by that of 16 ng guinea pig TG.

[^{3}H]putrescine-incorporation assay

TG activity was determined by measuring the incorporation of [^{3}H] putrescine into N,N′-dimethylcasein. The reaction mixtures (200 μl) contained 200 mM Tris-HCl, pH 7.5, 1 mg/ml of N,N′-dimethylcasein, 1 μCi of [^{3}H]putrescine, 2 mM $CaCl_2$, 10 mM dithiothreitol, 0.01% Triton X-100, 0.5 mM putrescine and vari-

ous concentrations of guinea pig TG (40 μl). Following incubation at 37°C for 20 min, the reaction was terminated with 200 μl of 10% trichloroacetic acid and spun down at 14,000 rpm for 20 min. The resulting pellets were suspended in 100 μl of 1.0 N NaOH and counted, after mixing with 100 μl of 1.0 N HCl, using a liquid scintillation counter.

Preparation of amine arrays

Glass slides (75 × 25 mm) were cleaned with a cleaning solution of $H_2O_2/NH_4OH/H_2O$ (1:1:5, v/v) at 70°C for 10 min, and sequentially washed with milli-Q water and with ethanol (Kim et al., 2006). The cleaned glass slides were incubated with 1.5% (v/v) 3-aminopropyltrimethoxysilane solution in 95% ethanol for 2 h, and washed with ethanol and with milli-Q water. The amine-modified glass slides were dried under compressed air and baked at 110°C for 1 h. Then, well-type amine arrays were fabricated by attaching teflon tapes (75 × 25 mm), each perforated with 108 holes (18 × 6) of 2.0 mm diameter, to the modified glass slides, and were stored under vacuum until use.

On-chip TG activity assay

The on-chip TG activity assay was performed on protein arrays as described in Fig. 1. To fabricate the protein arrays, the amine surfaces of the array wells were incubated with 1 mg/ml N,N′-dimethylcasein for 2 h at 37°C, and blocked with 3% bovine serum albumin containing 0.1% Tween 20 in a phosphate-buffered saline (PBS) (8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, pH 7.4, 2.7 mM KCl and 138 mM NaCl) The resulting protein arrays were washed with 0.1% Tween 20 in PBS and with milli-Q water. Reaction mixtures were prepared in 30 μl contained 200 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 1 mM 5-(biotinamido) pentylamine, 0.01% Triton X-100, 2 mM $CaCl_2$ and various concentrations of guinea pig TG or protein extracts. Then each 2 μl of the reaction mixtures was applied to the array wells at 37°C for the indicated time. The arrays were sequentially washed with 1% Tween-20 in PBS and with milli-Q water, and the TG-catalyzed incorporation of 5-(biotinamido) pentylamine into N,N′-dimethylcasein was probed with 100 μg/ml Cy3-conjugated streptavidin at 37°C for 1 h. Following washing with 1% Tween 20 in PBS and with milli-Q water, the arrays were dried under compressed air and scanned with a fluorescence scanner using a 532 nm laser (GSI Lumonics, Canada). The scanner was set to optimize the quality of the array images by adjusting the laser power. The fluorescence intensity of array spots was measured with the Fluoview program (Olympus, Japan), the fluorescence intensity representing the TG activity in the samples.

K_m determination

Each 2 μl of reaction mixtures containing various concentrations (2 to 100 μM) of 5-(biotinamido) pentylamine and 1 μg/ml guinea pig TG was applied to the casein surface of array wells at 37°C for 1 h. Then the arrays were probed with 100 μg/ml Cy3-conjugated streptavidin at 37°C for 1 h and analyzed with the fluorescence scanner. The K_m value of guinea pig TG for 5-(biotinamido) pentylamine was accessed with the Lineweaver-Burk double reciprocal plot.

Determination of TG activity

The fluorescence intensity of the array spots was measured with the Fluoview program (Olympus, Japan), the fluorescence intensity representing the TG activity in the samples. This TG activity was determined by the sigmoidal fit of the Origin program:

$$x = x_0[(A_1-A_2)/(y-A_2) - 1]^{1/p}, \quad (1)$$

where x is the TG activity of the samples, x_0 is the TG activity at half-maximal fluorescence intensity, A_1 is the minimal fluorescence intensity, A_2 is the maximal fluorescence intensity, y is the fluorescence intensity of the samples, and p is the power (the slope of the area about inflection point). The TG activity was expressed as nmol/min/mg protein.

Statistical analysis

The fit Linear function of the Origin program was used to calculate correlation coefficient (R^2). *t*-Test of two populations was used to evaluate the statistical significance of differences among groups.

RESULTS

Characterization of on-chip TG activity assay

For sensitive and high-throughput analysis of TG activity, we developed a novel assay system based on protein arrays, which system is illustrated in Fig. 1. We fabricated the protein arrays by immobilizing N,N′-dimethylcasein, as a TG substrate, on amine arrays, and applied directly reaction mixtures containing standard TG from guinea pig liver or cell extracts to the casein surface of the protein arrays. We then probed the resulting biotinylated casein with Cy3-conjugated streptavidin and determined the TG activity from the fluorescence intensities obtained by analyzing the protein arrays with a fluorescence scanner.

Since TGs are a Ca^{2+}-dependent family of enzymes (Griffin et al., 2002; Ruan and Johnson, 2007), we initially determined the optimal Ca^{2+} concentration for the on-chip TG activity assay. We prepared reaction mixtures containing various Ca^{2+} concentrations, applied the mixtures to protein arrays, and determined the TG activity with a fluorescence scanner. As shown in Fig. 2A, the TG activity showed a Ca^{2+}-dependent elevation with an evident activation at 200 μM, and it was almost saturated at 2 mM. Then, we determined the TG activity on the protein arrays according to the reaction time. As shown in Fig. 2B, the TG activity was linear from 15 to 120 min. However, in the absence of TG, fluorescence intensity was minimal at the indicated Ca^{2+} concentrations or at the indicated incubation times, indicating that fluorescence intensity on protein arrays was mostly contributed by TG activity. In addition, the TG activity was inhibited by cystamine and monodansylcadaverine, TG inhibitors, in a dose-dependent manner (data not shown).

Next, we evaluated reproducibility of the on-chip TG activity assay by analyzing inter-array, inter-reaction/-array and inter-spot reproducibility. Inter-array reproducibility was determined by analyzing the same batch of reaction mixtures on different arrays. We found the inter-array reproducibility to be high, with an average correlation coefficient of 0.990 (n = 3). Inter-reaction/-array reproducibility was also determined by analyzing different batches of reaction mixtures on different arrays. The inter-reaction/-array reproducibility was also high, with an average correlation coefficient of 0.988 (n = 3). In addition, inter-spot reproducibility was determined by analyzing ten replicate spots, and the average coefficient of variation was found to be 2.7% (n = 3). Taken together, these results demonstrate that the on-chip TG activity assay is highly reproducible.

We then determined sensitivity of the on-chip activity assay by analyzing TG activity of reaction mixtures containing various concentrations of guinea pig TG. As shown in Fig. 3A, in the presence of Triton X-100, the fluorescence intensity, representing the TG activity, increased in a TG-dose-dependent manner,

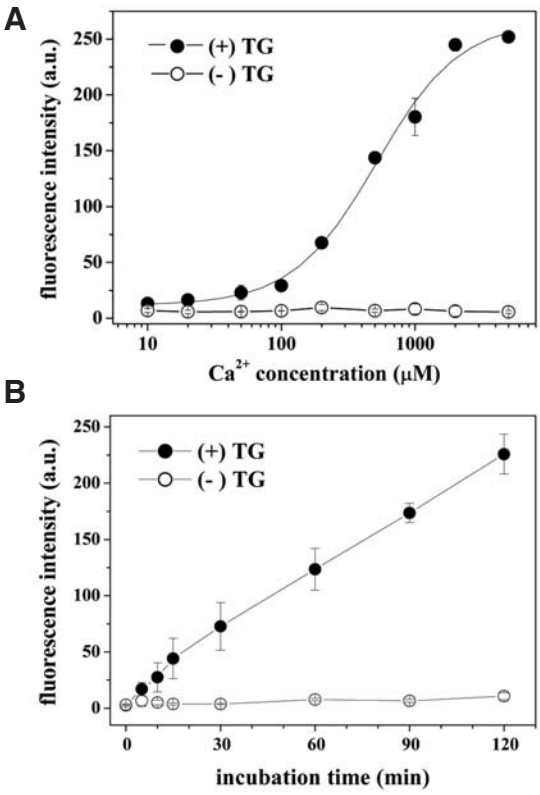

Fig. 2. Effects of Ca^{2+} concentration (A) and incubation time (B) on on-chip TG activity assay. Each 2 μl of reaction mixtures containing 1 μg/ml guinea pig TG was applied to the casein surface of protein arrays for 60 min in the presence of the indicated concentrations of Ca^{2+} (A) or for the indicated time in the presence of 2 mM Ca^{2+} (B). The resulting arrays were analyzed as described under Materials and Methods. The results are expressed as means ± S.D from three separate experiments.

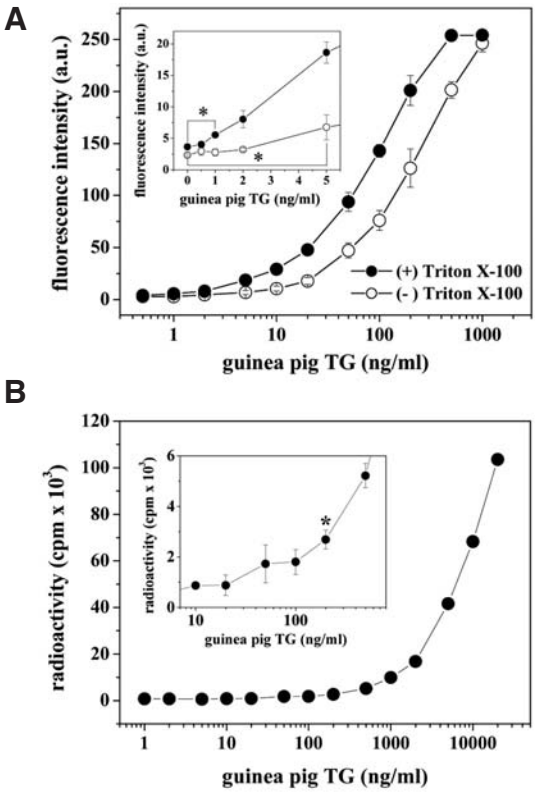

Fig. 3. Comparative determination of TG activity with on-chip TG activity assay and radioactive isotope-based putrescine-incorporation assay. (A) Reaction mixtures containing the indicated concentrations of guinea pig TG with or without Triton X-100 were applied in triplicate to the casein surface of protein arrays for 60 min and the arrays were probed with Cy3-streptavidin for 60 min. The arrays were then analyzed with a fluorescence scanner, after which the fluorescence intensities of the array spots were determined as described under "Materials and Methods". (B) Reaction mixtures containing 1 mg/ml N,N-dimethyl casein, Triton X-100 and [^{3}H]putrescine were incubated for 20 min in the presence of 2 mM Ca^{2+} and the indicated concentrations of guinea pig TG. The radioactivity of the resulting products was determined with a scintillation counter, as described under "Materials and Methods". The results are expressed as means ± S.D from three separate experiments (*p < 0.01).

significantly at 1 ng/ml ($p < 0.01$). However, in the absence of Triton X-100, the increase of the fluorescence intensity was significant at 5 ng/ml ($p < 0.01$), demonstrating that the TG activity was approximately five-times more sensitive in the presence of Triton X-100. As expected, in the absence of 5-(biotinamido) pentylamine, fluorescence was not detectable at any concentrations of guinea pig TG, demonstrating that the TG activity was mostly contributed by biotinylated casein. In addition, we determined K_m value of the on-chip TG activity assay with the Lineweaver-Burk double reciprocal plot, and the K_m value for 5-(biotinamido) pentylamine was 10.64 μM. Thus, we could conclude that the on-chip TG activity assay using protein arrays is highly sensitive and suitable for high-throughput determination of TG activity.

Analytical comparison of on-chip TG activity assay with [^{3}H]putrescine-incorporation assay

In order to compare the sensitivity of the on-chip TG activity assay with that of the previously used putrescine-incorporation assay, we performed the putrescine-incorporation assay with [^{3}H]putrescine, instead of 5-(biotinamido) pentylamine used for the on-chip assay, as a substrate. Initially, we determined the TG activity in various concentrations of Ca^{2+}. The TG activity increased in a Ca^{2+}-dependent manner, showing an apparent effect at 100 μM and effecting maximal stimulation at 2 mM, similar to the previous results obtained by the on-chip activity assay. That is, the two different TG activity assays showed a similar dependency on Ca^{2+} concentration.

Next, we performed the putrescine-incorporation assay in the presence of various concentrations of guinea pig TG. As shown in Fig. 3B, the TG activity was detectable at 200 ng/ml ($p < 0.01$), and then exponentially increased until 20,000 ng/ml, which results demonstrate that the on-chip TG activity assay was about 200 times more sensitive than the conventional putrescine-incorporation assay. Thus, again, we conclude that the on-chip TG activity assay based on protein arrays is a very sensitive and high-throughput approach for determining TG activity.

Application of on-chip TG activity assay to rapid analysis of TG activity in RA-treated mammalian cells

As it has been reported that RA induces TG2 expression in NIH 3T3 fibroblasts and human neuroblastoma SH-SY5Y cells (An

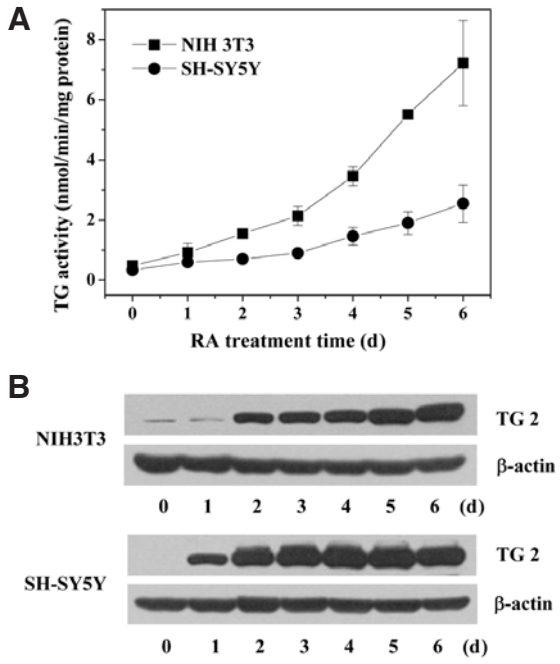

Fig. 4. Determination of TG activity by on-chip TG activity assay in RA-treated NIH 3T3 fibroblasts and SH-SY5Y neuroblastoma cells. (A) NIH 3T3 and SH-SY5Y cells were incubated with 10 μM RA for the indicated time (in days), and the cell extracts were mixed with the reaction cocktail for the on-chip TG activity assay and applied to the well-type protein arrays. The TG activity, expressed as nmol/min/mg protein, was determined as described under Materials and Methods. The results are expressed as means ± S.D from three separate experiments. (B) The expression level of TG2 determined by Western blotting.

tonyak et al., 2006; Tucholski and Johnson, 2003; Zhang et al., 1998), we applied the on-chip TG activity assay to the investigation of that expression. We initially treated NIH 3T3 cells with 10 μM RA for the indicated time, and we mixed cell extracts, in parallel with various concentrations of the TG standard, with the reaction cocktail, applying them in triplicate to the well-type protein arrays. We then analyzed the protein arrays using a fluorescence scanner. We plotted the fluorescence intensity of each standard sample as a function of the TG activity and obtained a standard curve from the plot by the Eq. (1). Then, we determined the TG activity in the cell extracts using the standard curve. As shown in Fig. 4A, the TG activity was detectable in the control NIH 3T3 cells, and increased according to the RA treatment time, with a maximal activation of about 7 nmol/min/mg protein at day 6, demonstrating that the on-chip TG activity assay is sensitive enough to study the expression of TG2 in control and RA-treated NIH 3T3 cells.

We then investigated the correlation between the TG activity and the TG2 expression level in the RA-treated NIH 3T3 cells. We analyzed the TG2 expression level by Western blotting (Fig. 4B), and scanned the resulting TG2 bands to express the relative amounts of the enzyme by normalized intensities (data not shown). TG2 expression was detectable at day 0, thereafter increasing according to the RA treatment time. However, neither TG1 nor TG3 were detectable in the control and RA-treated cells (data not shown). We analyzed the correlation between the TG2 expression level and the TG activity by determining the relative specific TG activity. We determined the relative specific TG activity by dividing the TG activity by the TG2 expression level. The specific activity at day 2 decreased to half of that at day 1 and then continuously increased until day 6 (data not shown).

In order to confirm the effectiveness of the on-chip TG activity assay, we utilized it to analyze the TG activity in neuroblastoma SH-SY5Y cells differentiated with 10 μM RA for 6 d. As expected from the previous reports (Tucholski and Johnson, 2003; Zhang et al., 1998), RA stimulated TG2 expression significantly at day 1, TG2 expression reaching its plateau at day 5 (Fig. 4B). The maximal TG2 expression level in the RA-stimulated SH-SY5Y cells was about 1.3-fold higher than that in the RA-treated NIH 3T3 cells, indicating that RA differentially induced the TG2 expression in different cells. TG1 and TG3 were not detected by Western blotting in the control and RA-treated neuroblastoma cells (data not shown). Next, using the on-chip TG activity assay, we determined the TG activity in RA-treated neuroblastoma cells (Fig. 4A). In the untreated control cells, as in the control neuroblastoma cells, the TG activity was detectable. However, the TG2 expression was not detectable in the control neuroblastoma cells, indicating that that the on-chip assay is more sensitive in determining TG activity than is Western blotting in determining TG2 expression level. Thereafter, the TG activity increased according to the RA treatment time, with a maximal stimulation of about 2.5 nmol/min/mg protein at day 6. These results demonstrated that the maximal TG activity in the RA-treated SH-SY5Y cells was lower than that in the RA-treated NIH 3T3 cells. Thus, RA induced a higher level of TG2 expression but a lower TG activity in SH-SY5Y cells than in NIH 3T3 cells, indicating differential regulation by RA in the expression and activation of TG in different cells. Therefore, the on-chip TG activity assay was confirmed to be a sensitive and effective system for studying the regulation of TG in cultured animal cells.

Monitoring of TG activity by on-chip TG activity assay in various mammalian cell lines

Since the previous results showed a successful analysis of TG activity in the two cell lines treated with RA, we further analyzed the TG activity in seven human cell lines (AGS, HeLa, HCT116, J82, BEAS-2B and SH-SY5Y cells) and four mouse cell lines (B16F10, Raw, Swiss 3T3 and NIH 3T3 cells). As shown in Fig. 5, the HUVECs and Raw cells showed the highest TG activity, whereas the lowest TG activity was observed in the J82, Swiss 3T3 and SH-SY5Y cells. However, the TG2 expression level was not correlated with the TG activity (data not shown). For example, the TG2 expression level of Raw cells is much lower than that of HUVECs. These results demonstrate the significance of activity-based analysis of TG, and moreover suggest that the on-chip TG activity assay is a sensitive system for studying TG in cultured mammalian cells.

In conclusion, we found the on-chip TG activity assay to be a novel, sensitive and high-throughput approach to monitoring TG activity in investigating its role(s) in cellular signaling.

DISCUSSION

In this paper, we present a novel on-chip TG activity assay using protein arrays to determine quantitative levels of TG activity in a parallel format. We prepared the protein arrays by immobilizing N,N′-dimethylcasein on amine-coated arrays. The on-chip TG activity assay was highly reproducible and shown to be much more sensitive than that of the conventional [^{3}H] putrescine-incorporation assay. We successfully applied the on-chip assay to a parallel analysis of the quantitative TG activity in

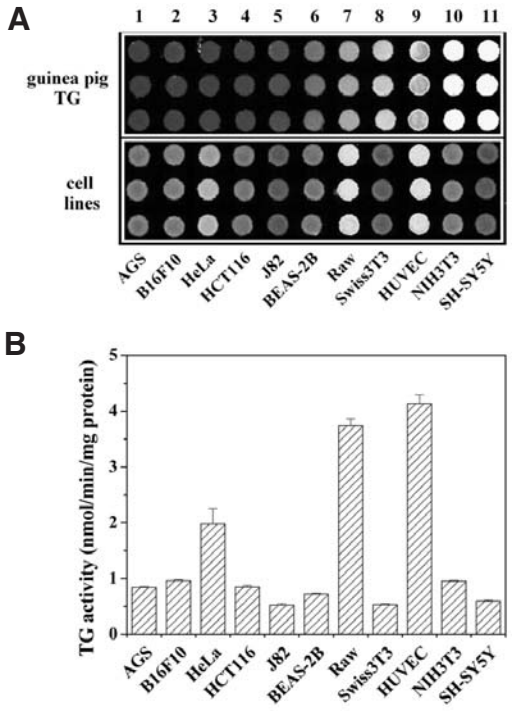

Fig. 5. Determination of TG activity by on-chip TG activity assay in cultured mammalian cells. Protein extracts from various mammalian cell lines, including AGS, B16F10, HeLa, HCT116, J82, BEAS-2B, Raw, Swiss 3T3, HUVEC, NIH 3T3, and SH-SY5Y, were loaded onto well-type protein arrays. Then, the arrays were analyzed with a fluorescence scanner (A) and the TG activity was determined as described under "Materials and Methods" (B). The guinea pig TG activities in (A) were 0, 0.02, 0.04, 0.1, 0.2, 0.4, 1, 2, 4, 10 and 20 nmol/min/ml, for 1 to 11, respectively. The results are expressed as means ± S.D from three separate experiments.

cultured eleven mammalian cell lines and RA-differentiated NIH 3T3 and SH-SY5Y cells.

To our understanding, this is the first report of a quantitative and high-throughput analysis of TG activity on protein arrays. TGs are involved in a number of functions such as cell regulation including apoptosis, neurodegenerative disorders, autoimmune diseases, inflammation, wound healing, cardiac and vascular diseases and tumor progression (Esposito and Caputo, 2005; Kim, 2006; Kotsakis and Griffin, 2007; Ruan and Johnson, 2007; Sane et al., 2007; Verderio et al., 2004). TG activity has been determined by a number of assays including polyamine-incorporation assays, photometric assays, and enzyme-linked colorimetric assays (Jeon et al., 2003; Wu and Tsai, 2006). However, there is no report on the protein array-based determination of TG activity in a high throughput manner. Development of simple, sensitive, radioactive isotope-free and parallel-format methods for determining TG activity has proved to be a considerable challenge. One new method through the on-chip TG activity assay has several advantages over the conventional methods. The on-chip activity assay doses not require stop solution because unbound substrates and fluorescent molecules are simply removed by washing the arrays. In addition, the assay does not require radioactive isotopes and is performed a very small sample format. Furthermore, the on-chip activity assay is simple and highly sensitive, compared to other assays. Thus, the on-chip TG activity assay can be considered to be a potential system for investigating signal transduction mechanisms as well as for diagnosis of human diseases.

It is the ultimate goal of proteomics to assign functions to networks of proteins in normal and pathological processes (Sadaghiani et al., 2007; Schmidinger et al., 2006). Global analysis of protein expression levels by abundance-based proteomic approaches does not provide direct information about protein functions, because protein expression levels might not correlate with protein activities. Protein activities are responsible for metabolic fluxes, cell regulation and signal transduction, and they are highly regulated by post-translational modifications such as phosphorylation, acylation, methylation and others (Schmidinger et al., 2006). In agreement with this report, in the present study, the TG2 expression level was higher in SH-SY5Y cells, whereas the specific TG activity was lower in the neuroblastoma cells. In addition, in the case of RA-treated neuroblastoma cells, activity-based analysis was more sensitive than expression-based analysis. Therefore, activity-based proteomics can overcome the limitation of abundance-based proteomics (Sadaghiani et al., 2007; Schmidinger et al., 2006).

Recently, whereas there have been an accumulating number of papers on the high-throughput analysis of protein phosphorylation and protein kinase activity, reports on the activity analysis of non-kinase enzymes have remained limited in number. Protein arrays have been applied to analyze the activity of protein kinases or protein phosphorylation using radioactive isotopes and fluorophores (Houseman et al., 2002; Martin et al., 2003; Zhu et al., 2000). Array-based activity assay of protein kinases at the proteome level has been demonstrated by Zhu et al. (2000), who characterized, with 17 different substrates on microwells, 119 protein kinases isolated from yeasts. Substrate identification of protein kinases has been performed on peptide or protein arrays, including protein kinase A, casein kinase II, p42 mitogen-activated protein kinase and c-src (Houseman et al., 2002; MacBeath and Schreiber, 2000; Shigaki et al., 2007). It has been reported that a small molecule fluorescence phosphosensor, Pro-Q diamond dye, was used for the analysis of phosphorylated amino acids in peptides and proteins displayed on arrays (Martin et al., 2003), and there have been recent reports on global profiling of protein phosphorylation based on reverse phase protein arrays (Machida et al., 2007; Ptacek et al., 2005). However, there remain only a limited number of reports on the array-format activity analysis of phosphorylation-independent enzymes. Thus, the on-chip activity assay can provide a potential approach to the investigation of enzyme functions at the proteome level.

In summary, we developed an on-chip TG activity assay based on well-type protein arrays to determine the quantitative level of TG activity in a parallel format. This new approach is simple, sensitive and high-throughput, and thus has a strong potential for use in cell research and activity-based proteomics as well as diagnosis of human diseases such as celiac disease.

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