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Transglutaminase down-regulates the dimerization of epidermal growth factor receptor in rat perivenous and periportal hepatocytes

A. Maruko*, Y. Ohtake*, S. Katoh† and Y. Ohkubo*

**Department of Radiopharmacy, Tohoku Pharmaceutical University, Komatsushima, Aoba-ku, Sendai, Miyagi, Japan, †Department of Life Science, Laboratory of Radiophysiology, Yokohama College of Pharmacy, Matano-cho, Totsuka-ku, Yokohama, Japan*

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Abstract

Objective: Recently, we found that transglutaminase 2 (TG2) might be involved in the difference in proliferative capacities between periportal hepatocytes (PPH) and perivenous hepatocytes (PVH) through down-regulation of high-affinity epidermal growth factor receptor (EGFR). However, it is uncertain whether this high-affinity EGFR contributes to the hepatocyte growth signalling pathway. Here, we have investigated the influence of TG2 on EGF-induced EGFR dimerization and its phosphorylation, which are important steps in the hepatocyte proliferative/growth signalling pathway, in PPH and PVH.

Materials and methods: PPH and PVH were isolated using the digitonin/collagenase perfusion technique. Amounts of TG2, EGFR dimerization and its phosphorylation were determined by Western blot analysis.

Results: Pretreatment with monodansylcadaverine, an inhibitor of TG2, greatly increased EGF-induced EGFR dimerization and its phosphorylation in PVH compared with PPH. Conversely, treatment with retinoic acid, an inducer of TG2, significantly decreased EGF-induced EGFR dimerization and its phosphorylation with a significant increase in TG2 expression and its catalysed products, isopeptide bonds, in both subpopulations. It was found that EGFR served as a substrate for TG2.

Conclusion: The present data showed good correlation with our previous data on EGF-induced DNA synthesis and EGFR-binding affinity to EGF. These results suggest that zonal difference in cell growth between PPH and PVH may be caused by down-regulation of EGFR dimerization and subsequent autophosphorylation through TG2-mediated cross-linking of EGFR.

Correspondence: Y. Ohtake, Department of Radiopharmacy, Tohoku Pharmaceutical University, 4-4-1, Komatsushima, Aoba-ku, Sendai, Miyagi 981-8558, Japan. Tel: +81 22 727 0121; Fax: +81 22 275 2013; E-mail: y-ohtake@tohoku-pharm.ac.jp

Introduction

Hepatocytes are classified into periportal hepatocytes (PPH) and perivenous hepatocytes (PVH, central vein) on the basis of previous studies, which have demonstrated their zonal differences in metabolism (1,2) and proliferation (3). Using an experimental model of hepatocyte proliferation, induced by 70% partial hepatectomy, PPH and PVH show different growth capacities, and DNA synthesis in PPH is greater than that in PVH (4,5). In a primary culture system, PPH and PVH show different responses to various mitogens, such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (6,7). However, molecular mechanisms underlying the zonal differences in proliferative response are not well understood.

Transglutaminase 2 (TG2; EC 2.3.2.13) is a member of a family of enzymes that catalyses a calcium-dependent transamidation reaction which results in protein–protein association through the formation of ε-(γ-glutamyl) lysine isopeptide bonds (8,9). Melino *et al*. demonstrated that TG2 overexpression caused a dramatic reduction in proliferative capacity of human neuroblastoma cells (10). We have reported that inhibition of *de novo* synthesis of TG2 resulted in increased growth of normal rat hepatocytes in the presence of EGF (11) or HGF (12). Recently, we reported that PPH and PVH proliferation are inversely correlated with DNA synthesis and TG2 activity during liver regeneration after partial hepatectomy, suggesting that TG2 regulates the growth signal (4). Moreover, we have reported that TG2 is involved in the difference in growth capacities between PPH and PVH through down-regulation of high-affinity EGF receptors (EGFR) (13). EGFR normally falls into two subpopulations, the majority being low-affinity (95–98%) and the minority being

high-affinity (2–5%) class receptors (14). It has been reported that the affinity classes represent different receptor conformations; the monomeric tethered configuration is equivalent to low-affinity receptors, whereas the extended configuration is equivalent to high-affinity receptors (15,16). It has been demonstrated that high-affinity EGFR may play an important role in EGF-induced cell proliferation (14,17). Furthermore, binding of EGF to the receptor causes dimerization between EGFRs and induces phosphorylation of tyrosine residues on the receptor and thereafter leads to activation of extracellular signal-regulated kinase, resulting in induction of cell growth (18). Therefore, EGFR dimerization and its phosphorylation must also play an important role in EGF-induced cell growth signal transduction.

It is possible that TG2 affects EGFR dimerization and its phosphorylation through down-regulation of high-affinity EGFR, resulting in zonal differences in cell growth between PPH and PVH.

In the present study, to test this possibility, we investigated the influence of TG2 activity on EGF-induced EGFR dimerization and its phosphorylation in primary cultured PPH and PVH.

Materials and methods

Materials

[Methyl-^{3}H] thymidine was purchased from PerkinElmer Life Sciences Inc. (Boston, MA, USA). Collagenase was obtained from Nitta Gelatin (Osaka, Japan). Digitonin, monodansylcadaverine (MDC), and anti-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse EGF was obtained from Biomedical Technologies Inc. (Stoughton, MA, USA). Retinoic acid was obtained from Wako (Osaka, Japan). Anti-EGFR polyclonal antibody, anti-phospho-EGFR antibody, specific to residue Y1173, and goat anti-rabbit immunoglobulin G antibody conjugated with peroxidase were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-TG2 antibody was obtained from Neomarker (Fremont, CA, USA). Ne (g-L-glutamyl)-L-lysine (Gln-Lys) isopeptide mouse monoclonal antibody (81D4) was obtained from Covalab (Villeurbanne, France). All other reagents were readily available commercial products of analytical grade and were used without further purification.

Animals

Male Wistar rats weighing 200–230 g (SLC, Hamamatsu, Japan) were kept in a controlled temperature (23 ± 1 °C) under a 12-h light–dark cycle and were fed with standard diet and water. All animal experiments were performed in strict accordance with our institutional animal committee's criteria for the care and use of laboratory animals.

Isolation and culture of PPH and PVH

PPH and PVH subpopulations were isolated using the digitonin/collagenase perfusion technique described by Quistroff (19) with modifications as described by Imai *et al*. (7). The detailed procedure has been described previously (4). After perfusion with collagenase solution, the liver lobes were carefully transferred to Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) and minced gently, then the tissue was filtered through a 100-μm-nylon mesh. After centrifugation at 50 ***g*** for 2 min, supernatant was removed, and cells were resuspended in DMEM and recentrifuged. After this procedure was repeated three times, cells were suspended in William's E medium (Sigma-Aldrich, St. Louis, MO, USA). Viability was determined by trypan blue staining and was found to be at a level of more than 90% at this point. PPH and PVH were placed in 12-well collagen-coated plates (Iwaki, Tokyo, Japan) at a density of 0.8×10^5 cells/cm^2 in William's E medium containing 10% foetal bovine serum, 10^{-9} M insulin, 10^{-9} M dexamethasone and 1% (v/v) antibiotics [penicillin G sodium (100 U/ml), streptomycin sulphate (100 μg/ml), amphotericin B (0.25 μg/ml) (Gibco, Grand Island, NY, USA)]. After 3 h incubation at 37 °C in an atmosphere of 95% air and 5% CO_2 at 100% relative humidity, medium was replaced with serum-free medium.

Pretreatment of hepatocytes with MDC or retinoic acid

In the case of MDC treatment, after attachment, cells were cultured with serum-free medium for 24 h, and were then pretreated for 30 min with MDC (0.5 mM). After the cells had been washed three times with serum-free medium, EGF was added. In the case of retinoic acid treatment, after attachment, cells were incubated for 24 h with retinoic acid (5 μM), and then EGF was added. Thereafter, cells were subjected to the following experimental procedures.

Measurement of DNA synthesis

DNA synthesis was assessed by [methyl-^{3}H] thymidine incorporation into hepatocytes. [Methyl-^{3}H] thymidine (0.5 μCi/ml) incorporation in PPH and PVH from 48 to 72 h was measured after treatment with or without 10^{-8} m EGF, a concentration that induced maximum proliferative effect. After incubation with or without EGF at 72 h, cells were washed in phosphate-buffered saline (PBS) (pH 7.4), and solubilized in 1 m NaOH. Radioactivity of [methyl-^{3}H] thymidine incorporated into cells was measured using a

Beckman LS 6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA). DNA synthesis in hepatocytes was determined from incorporation of [methyl-^{3}H] thymidine expressed in disintegration/min/mg protein (dpm/mg protein). Protein concentration was determined according to the method of Bradford using bovine serum albumin as standard (20).

Cross-linking of EGFRs

To determine EGFR dimerization, EGFR cross-linking was carried out as follows. After treatment with EGF at intervals 1 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, and 6 h, hepatocytes were washed twice in PBS. They were then incubated for 1 h at 4 °C with cross-linker, bis (sulphosuccinimidyl) suberate (BS^3) (Pierce, Rockford, IL, USA) dissolved to final concentration of 2 mM, in PBS. Quench solution (0.5 M Tris-HCl buffer, pH 7.4) was then added to a final concentration of 0.25 M and incubated for 10 min at 4 °C. Hepatocytes were then washed twice in PBS, and were subjected to hepatic membrane fractionation.

Fractionation of hepatocyte membrane and cytosol

Cultured hepatocytes were homogenized in 50 mM Tris-HCl buffer containing 1 mM EDTA, and 10% glycerol supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich) and 1% phosphatase inhibitor cocktail (Sigma-Aldrich) with 30 strokes on a Dounce homogenizer. Homogenates were then centrifuged at 500 ***g*** for 5 min and supernatant was collected and centrifuged at 100 000 ***g*** for 30 min. The pellet was used as membrane fraction, and an aliquot of the resultant supernatant was used as cytosol fraction, for Western blot analysis.

Determination of TG2 expression

To determine TG2 protein expression, cultured hepatocytes were lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40) containing 1% protease inhibitor cocktail for 1 h at 4 °C. The cell lysate was then centrifuged at 15 000 ***g*** for 5 min. The supernatant was subjected to Western blot analysis.

Immunoprecipitation

Cultured hepatocytes were lysed in buffer composed of 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 10% glycerol and 1% protease inhibitor cocktail, and cell debris was removed by centrifugation at 15 000 ***g*** for 5 min. Cell lysates were incubated overnight with anti-EGFR antibody at 4 °C. Immunocomplexes were precipitated with protein G-sepharose (GE Healthcare, Buckinghamshire, England), the immunoprecipitates were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and to subsequent Western blotting using anti-isopeptide antibody.

Western blot analysis

Samples were mixed with sample buffer for SDS-PAGE containing 62.5 mM Tris-HCl buffer (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.025% bromophenol blue. The mixture was boiled at 100 °C for 3 min and was size-separated by SDS-PAGE on 3–10% polyacrylamide gradient gel (Bio-Rad, CA, USA). Separated membrane or lysate proteins were transferred on to polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, England) and immunoblot analysis was carried out using rabbit anti-TG2 antibody (final 5 μg/ml), rabbit anti-EGFR antibody (final 1 μg/ml), rabbit anti-phospho-EGFR antibody (final 0.1 μg/ml), rabbit anti-actin antibody (final 1 μg/ml) and goat anti-rabbit immunoglobulin G antibody conjugated with peroxidase. Immunoreactive bands were detected with ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, England) and exposed using X-ray film (Fujifilm, Tokyo, Japan).

Statistical analysis

Student's *t*-test and one-way analysis of variance were used for statistical analysis of [methyl-^{3}H] thymidine incorporation. For immunodetection analysis of TG2 or isopeptide, the Student's *t*-test was performed.

Results

Effects of MDC and retinoic acid on EGF-induced DNA synthesis in primary cultured PPH and PVH

PPH and PVH, isolated by the digitonin/collagenase perfusion technique, were identified by measuring activities of specific marker enzymes, alanine aminotransferase for PPH and glutamine synthetase for PVH. Activities of alanine aminotransferase and glutamine synthetase in PPH and PVH were consistent with previous reports (13), and in good agreement with published values (21), showing successful isolation for PPH and PVH (data not shown).

Results of DNA synthesis induced by EGF treatment in primary cultured PPH and PVH at 72 h were the same as those of previous reports (13). In PPH, MDC treatment increased EGF-induced DNA synthesis to approximately 1.5 times the control level. In PVH, MDC treatment greatly increased EGF-induced DNA synthesis to approximately 2.8 times the control level. Retinoic acid treatment decreased

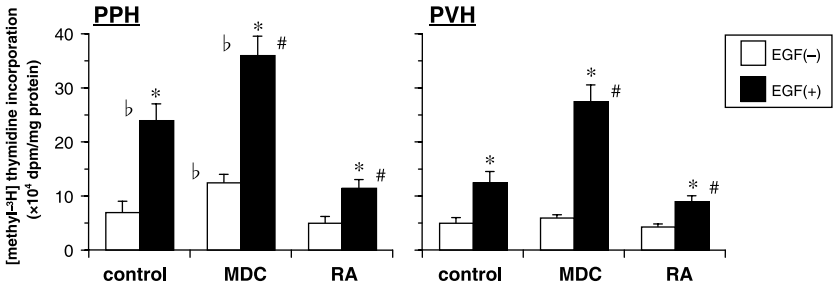

Figure 1. Effect of monodansylcadaverine (MDC) and retinoic acid (RA) on DNA synthesis induced by epidermal growth factor (EGF) in primary cultured periportal hepatocytes (PPH) and perivenous hepatocytes (PVH). Hepatocytes were pretreated with MDC (0.5 mM) for 30 min, or treated with RA (5 μM). DNA synthesis induced by EGF (10^{-8} m) treatment was measured by [methyl-^{3}H] thymidine incorporation method as described in the Materials and methods section. [Methyl-^{3}H] thymidine was induced from 48 to 72 h. Each value represents the mean ± standard error of the mean of four to five samples. $^*P < 0.05$, vs. respective EGF-untreated group by unpaired two-tailed Student's *t*-test. $^\#P < 0.05$, vs. EGF alone control by one-way analysis of variance. $^bP < 0.05$, compared to PPH with PVH by unpaired two-tailed Student's *t*-test.

EGF-induced DNA synthesis in PPH to approximately one-half of the control level, whereas in PVH, retinoic acid-mediated reduction of DNA synthesis was clearly < 50% (Fig. 1). In order to determine whether the observed stimulation of DNA synthesis resulted from a replication or from a repair process, we examined the inhibitory effect of aphidicolin, a specific inhibitor of the replication enzyme, DNA polymerase α. Addition of 10 ng/ml of aphidicolin to cultured hepatocytes completely abolished EGF-stimulated [methyl-^{3}H] thymidine incorporation without any effect on cell viability (data not shown).

Effects of MDC and retinoic acid on TG2 expression in primary cultured PPH and PVH

To investigate of the effects of MDC and retinoic acid on TG2 expression, Western blot analysis using a TG2 antibody was performed on cell lysates from hepatocytes treated with MDC or retinoic acid.

As shown in Fig. 2, in MDC-treated cells of both subpopulations, TG2 expression was the same as control levels. By comparison, in retinoic acid-treated cells, TG2 expression in PPH and PVH markedly increased by about 2 and 1.5 times the control level, respectively. Overall, level of TG2 expression in PVH was about 1.5–2 times higher than that in PPH.

Detection of substrates for TG2-catalysed cross-linking reaction

To demonstrate that EGFR could serve as a TG2 substrate, we performed immunoprecipitation with anti-EGFR antibody and subsequent Western blotting with anti-Gln-Lys isopeptide antibody. As shown in Fig. 3, the distribution of approximately 170-kDa cross-linked proteins recognized by the isopeptide antibody was observed. Therefore, the

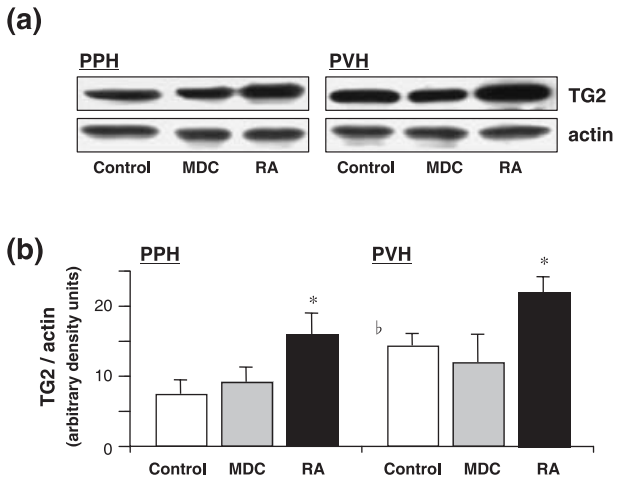

Figure 2. Effect of monodansylcadaverine (MDC) and retinoic acid (RA) on transglutaminase 2 (TG2) expression in primary cultured periportal hepatocytes (PPH) and perivenous hepatocytes (PVH). Cultured hepatocytes were treated with or without MDC (0.5 mM) or RA (5 μM) as described in the Materials and methods section, then hepatocytes were lysed, and analysed for TG2 level by Western blot analysis using an anti-TG2 antibody. Band quantitation was performed with National Institutes of Health Image software. (a) Immunodetection of TG2 and actin are shown. Results represent one typical experiment. (b) The histogram represents mean ± standard error of the mean of three independent experiments, expressed TG2/actin quantitated results presented in arbitrary density units. $^*P < 0.05$, vs. respective control group by unpaired Student's *t*-test. $^bP < 0.05$, compared to PPH with PVH by unpaired two-tailed Student's *t*-test.

result demonstrates that EGFR had served as a good substrate for TG2. In control cells, formation of isopeptide bonds in EGFR in PVH was slightly higher than that in PPH. In MDC-treated cells of both subpopulations, levels of Gln-Lys isopeptide bonds in EGFR were decreased. By comparison, in retinoic acid-treated cells of both subpopulations, the levels were greatly increased. In all

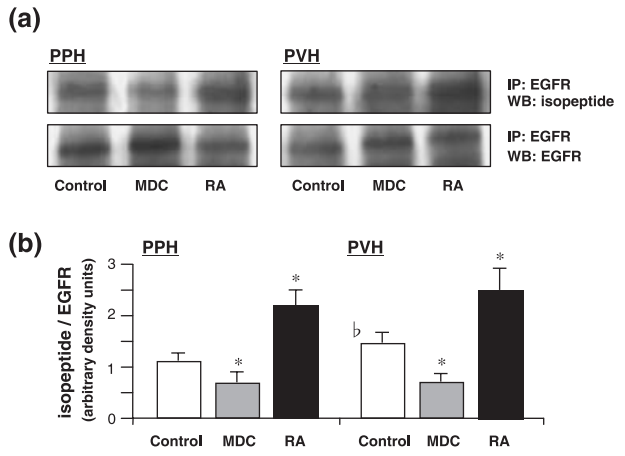

Figure 3. Effect of monodansylcadaverine (MDC) and retinoic acid (RA) on isopeptide cross-link formation in primary cultured periportal hepatocytes (PPH) and perivenous hepatocytes (PVH). Cultured hepatocytes were treated with or without MDC (0.5 mM) or RA (5 μM) as described in the Materials and methods section, than hepatocytes were lysed, and epidermal growth factor receptor (EGFR) was immunoprecipitated (IP). Band quantitation was performed with National Institutes of Health Image software. (a) Isopeptide cross-linking formation of EGFR was analysed by Western blot analysis, using anti-EGFR antibody (upper panel) and anti-isopeptide antibody (lower panel). (b) The histogram represents mean ± standard error of the mean of three independent experiments, expressed isopeptide/EGFR quantitated results presented in arbitrary density units. $^{*}P < 0.05$, vs. respective control group by unpaired Student's *t*-test. $^{b}P < 0.05$, compared to PPH with PVH by unpaired two-tailed Student's *t*-test.

cases, EGF treatment showed no changes in formation of isopeptide bonds in EGFR (data not shown). Data showed good correlation between TG2 expression and TG2-catalysed cross-linking products, Gln-Lys isopeptide bonds.

Effects of MDC and retinoic acid on EGFR dimerization in primary cultured PPH and PVH

The effects of MDC and retinoic acid on EGFR dimerization at the cell membranes obtained from hepatocytes are shown in Fig. 4. Addition of EGF-induced redistribution of the receptor from the 170–175 kDa band to the 340–350 kDa band corresponding to monomeric and dimeric forms of EGFR, respectively. Hepatic membranes from cultured hepatocytes without EGF treatment were used as the control for EGFR activation (lane 1). As shown in Fig. 4, dimerization of EGFRs in PPH and PVH increased in response to EGF stimulation. Maximum dimerization was achieved within 5 min following EGF treatment, was sustained at this level for 30 min and thereafter decreased. Dimerization of EGFRs following EGF treatment was greater in PPH than in PVH. The effect of MDC treatment on EGF-induced EGFR dimerization was studied and in PPH, MDC treatment increased EGF-induced EGFR dimerization to approximately twice the level of MDC-untreated cells. In PVH, MDC treatment markedly increased EGF-induced EGFR dimerization to approximately 2–2.3 times the level of non-MDC-treated cells. MDC treatment caused the rate of dimerization in PVH to reach a value similar to that in PPH. Maximum dimerization was achieved within 5 min following EGF treatment and was sustained for 1 h. By comparison, retinoic acid treatment significantly decreased dimerization of EGFRs in both subpopulations, and rate of dimerization between PPH and PVH was not significantly different.

Effects of MDC and retinoic acid on cytosolic EGFR level in primary cultured PPH and PVH

In experiments with EGFR dimerization (Fig. 4), strong increase in EGFR dimers following EGF treatment was observed, which was not paralleled by a decrease in monomers. It is expected that dimer-forming EGFRs are recruited from the EGFR monomer pool resulting in decrease of EGFR monomers. However, remarkable changes in EGFR monomer levels were not observed. Hence, we speculated that externalization of receptors from an intracellular pool might be involved. We next investigated the effects of MDC and retinoic acid on EGFR level in cytosolic fractions. Cytosolic fractions from cultured hepatocytes without EGF treatment were used as the control for EGFR level (Fig. 5, lane 1). As shown in Fig. 5, cytosolic EGFR levels in both subpopulations decreased in response to EGF stimulation. EGFR levels transiently decreased within 10 min following EGF treatment and returned to basal levels. Decreased rate of cytosolic EGFR level in PPH following EGF treatment was slightly greater than that in PVH. MDC treatment enhanced EGF-induced cytosolic EGFR decrease to approximately 40% of the level of non-MDC-treated cells in both subpopulations. By comparison, retinoic acid treatment showed no significant change in cytosolic EGFR level in both subpopulations. These results suggest that EGFR monomers on membranes are recruited from an intracellular receptor pool at the time of EGF stimulation.

Effects of MDC and retinoic acid on EGFR phosphorylation in primary cultured PPH and PVH

The effects of MDC and retinoic acid on EGFR phosphorylation at Y1173 of cell membranes obtained from hepatocytes are shown in Fig. 6. Phosphorylation of EGFR was examined in membranes of cultured hepatocytes from various time points following EGF treatment. As the control for EGFR phosphorylation, membranes from cultured hepatocytes without EGF treatment were used (lane 1). In MDC-treated cells of both subpopulations,

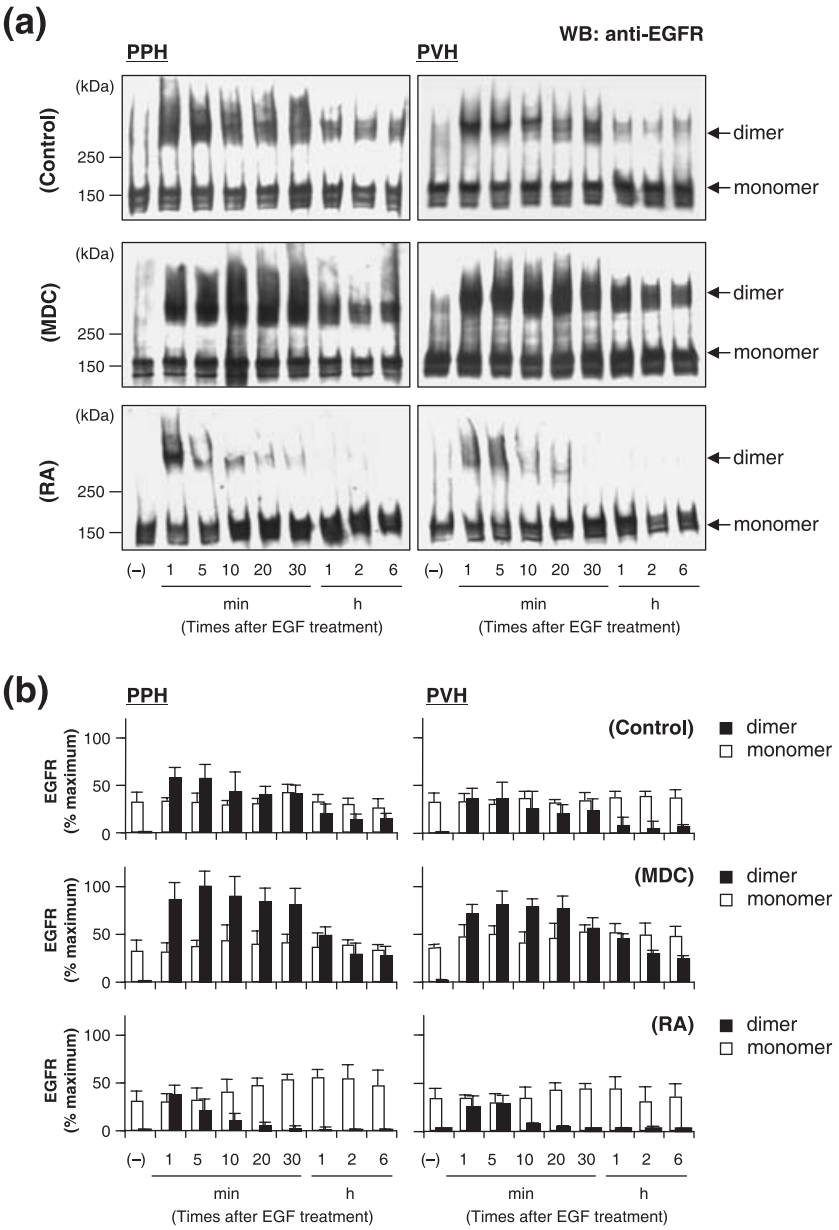

Figure 4. Effect of monodansylcadaverine (MDC) and retinoic acid (RA) on epidermal growth factor receptor (EGFR) dimerization induced by EGF treatment in primary cultured periportal hepatocytes (PPH) and perivenous hepatocytes (PVH). After EGF (10^{-8} m) treatment at intervals 1 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, and 6 h, proteins were cross-linked with bis-(sulphosuccinimidyl) suberate (BS^3). Cell membranes obtained from hepatocytes were then lysed and protein samples were immunoblotted with anti-EGFR antibodies. Band quantitation was performed with National Institutes of Health Image software. (a) Immunodetections of EGFRs (monomer and dimer) are shown. Results represent one typical experiment. Molecular weight markers are indicated on the left. (b) The histogram represents mean ± standard error of the mean of three independent experiments, expressed relative to the peak of EGFR dimer in PPH from MDC treated at 5 min following EGF treatment taken as 100%.

levels of EGFR phosphorylation significantly increased. By comparison, in retinoic acid-treated cells of both subpopulations levels of phosphorylation decreased to approximately half that of controls. Results of EGFR phosphorylation in MDC- and retinoic acid-treated hepatocytes showed good correlation with results of EGFR dimerization.

Discussion

Numerous studies have provided evidence that PPH and PVH show different proliferative/growth capacities. However, the molecular basis of this zonal difference in proliferative response is not well understood. Recently, we have reported that TG2 may be involved in differential growth capacities of PPH and PVH through down-regulation of high-affinity EGFR (13). Here, we report that TG2 affects EGF-induced EGFR dimerization and its subsequent autophosphorylation, which are important steps in the hepatocyte growth signalling pathway, and thereby zonal differences in cell proliferation between PPH and PVH occur.

We have previously reported that pretreatment with MDC, an inhibitor of TG2, greatly increased EGF-induced DNA synthesis, especially in PVH (13). Conversely, retinoic

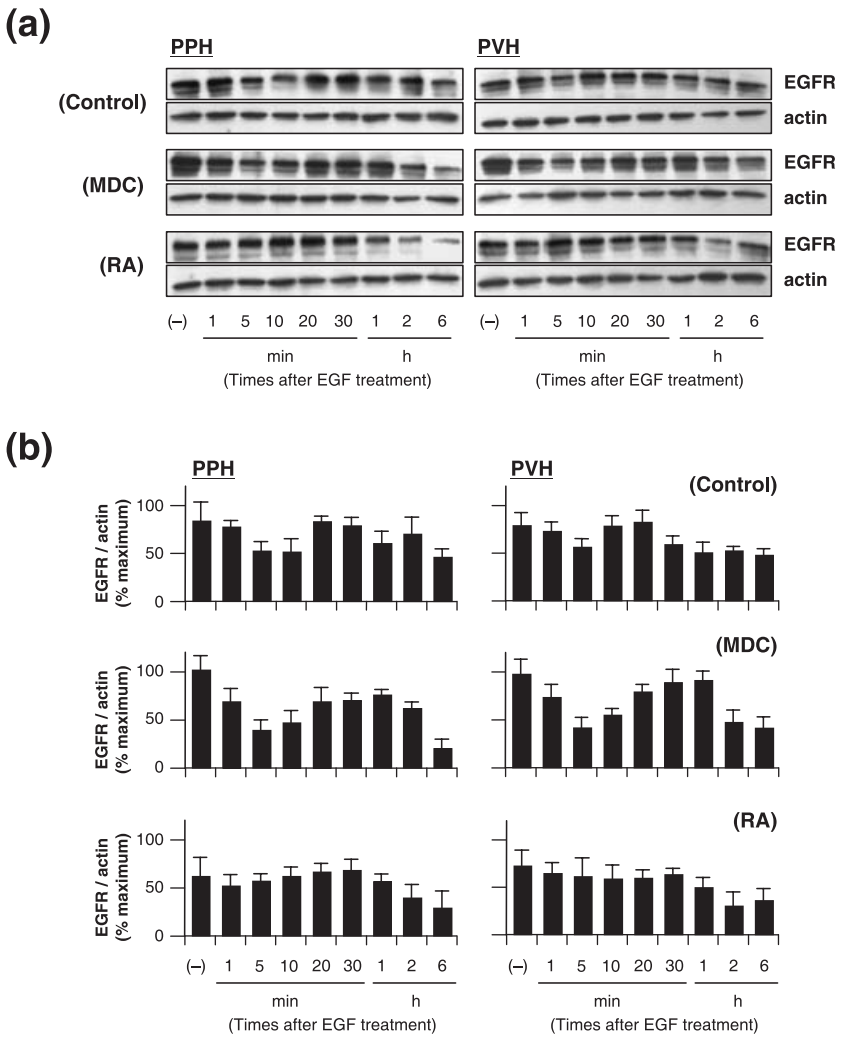

Figure 5. Changes in cytosolic epidermal growth factor receptor (EGFR) level following EGF stimulation. After EGF (10^{-8} m) treatment for indicated times, the cytosols obtained from cultured hepatocytes were immunoblotted with antibodies to EGFR. Band quantitation was performed with National Institutes of Health image software. (a) Immunodetections of EGFR and actin are shown. Results represent one typical experiment. (b) The histogram represents mean ± standard error of the mean of three independent experiments, expressed relative to the peak of the phosphorylation in EGFR in periportal hepatocytes (PPH) from monodansylcadaverine (MDC) treated at 5 min following EGF treatment taken as 100%.

acid, an inducer of TG2, significantly decreased EGF-induced DNA synthesis in both subpopulations (Fig. 1). These data suggest that TG2 negatively regulates EGF-induced cell growth signal. Thus, to investigate correlation between TG2 level and EGF-induced DNA synthesis, amounts of TG2 were examined in cultured PPH and PVH (Fig. 2). As a result, in control cells, the amount of TG2 in PPH was lower than that in PVH. This lower value of TG2 in PPH may lead to higher DNA synthesis than PVH through regulation of EGF-induced cell growth signals. On the other hand, in MDC-treated cells, no change in the amount of TG2 was observed in either subpopulation compared to control cells (Fig. 2). MDC is a primary alkyl amine with a fluorescent dansyl group attached at one end. Therefore, MDC, which is structurally similar to the lysine side chain, acts as a competitive substrate to inhibit TG2-mediated cross-linking of the natural substrate (22). As a result, there might be no change in the amount of TG2 in both subpopulations. In contrast, retinoic acid treatment resulted in significant increase in the amount of TG2 in both subpopulations (Fig. 2). However, it is not certain whether *de novo* synthesized TG2, by retinoic acid treatment, indicates biological activity, as the inactive form of TG2 is still present (23). As this point, we reported that retinoic acid-induced TG2 activity and its products, ε-(γ-glutamyl)lysine isopeptide bonds, were induced in partial-hepatectomized rat liver (24). Therefore, we think that the amount of TG2 detected by immunoblot analysis reflects biological activity occurring. Indeed, in immunoprecipitation experiments, TG2-catalysed cross-linking products, Gln-Lys isopeptide bonds, showed good correlation with TG2 expression. In the present study, we demonstrated for the first time that EGFR could be a substrate of TG2. However, the isopeptide antibody (81D4) used here reacts not only with Gln-Lys but also with bis(γ-glutamyl) polyamine. Therefore, polyamines such as putrescine, spermidine and spermine, which are ubiquitous polycationic compounds,

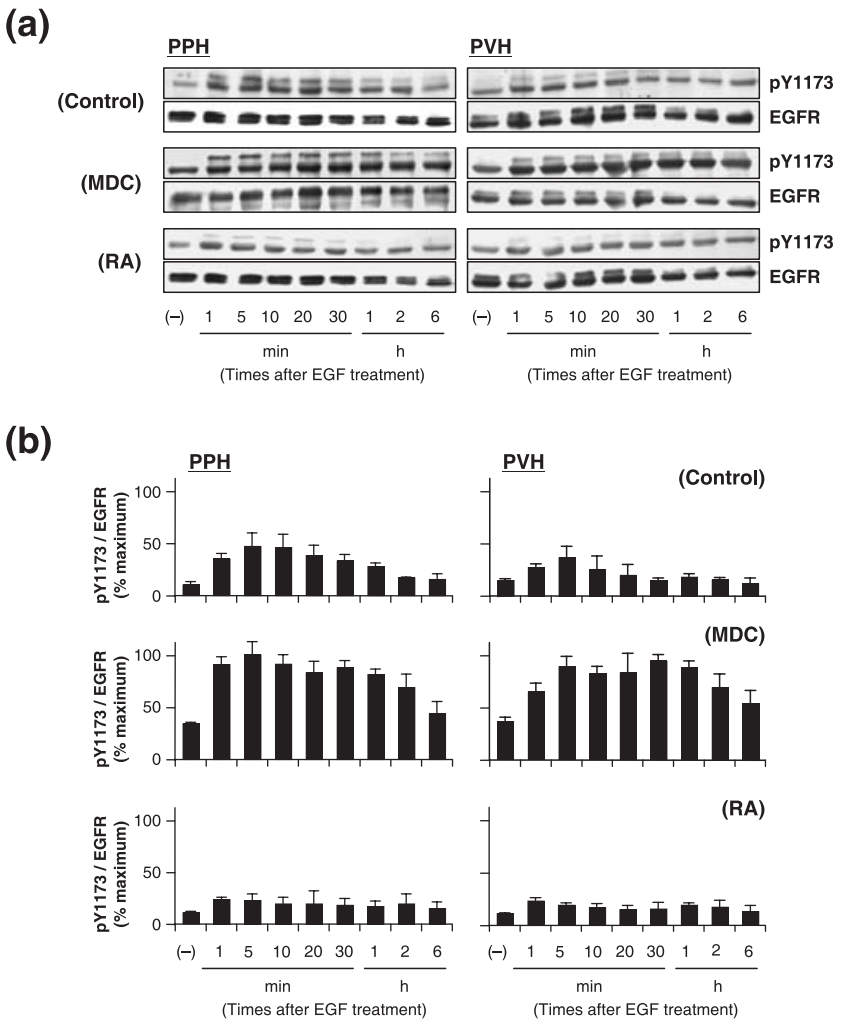

Figure 6. Effect of monodansylcadaverine (MDC) and retinoic acid (RA) on epidermal growth factor receptor (EGFR) phosphorylation induced by EGF treatment in primary cultured periportal hepatocytes (PPH) and perivenous hepatocytes (PVH). After EGF (10^{-8} m) treatment for indicated times, cell membranes obtained from cultured hepatocytes were lysed and protein samples were immunoblotted with antibodies to EGFR phosphorylated at Y1173 and EGFR. To determine specific tyrosine phosphorylation of EGFR, PVDF membranes proved with anti-phosphotyrosine antibody were stripped with a solution of 0.1 m glycine (pH 2.1) and reprobed with anti-EGFR antibody. The band quantitation was performed with National Institutes of Health image software. (a) Immunodetections of EGFR phosphorylation and EGFR are shown. Results represent one typical experiment. (b) The histogram represents mean ± standard error of the mean of three independent experiments, expressed relative to the peak of the phosphorylation in EGFR in PPH from MDC treated at 5 min following EGF treatment taken as 100%.

may be involved in TG2-catalysed intramolecular cross-linking of EGFR.

In a previous report, we have shown that inhibition of *de novo* synthesis of TG2 resulted in increased ratio of S-phase rat hepatocytes in the presence of EGF (11) or HGF (12). Moreover, we found that the preferential *de novo* synthesis of TG2 in PVH as compared with PPH is induced by partial hepatectomy, leading to a lower rate of DNA synthesis in PVH (4). Considering the results of previous reports in addition to the present one, it is possible that TG2 regulates differences in cell growth between PPH and PVH. However, MDC, an inhibitor of TG2, treatment did not completely abolish these differences (Fig. 1) and several factors may be involved in TG2-mediated differences in zonal proliferation.

Binding of EGF to the extracellular domain of its receptor causes dimerization of EGFRs, resulting in activation of its downstream signalling pathways. In the present study, in control cells, EGF-induced dimerization of EGFR was greater in PPH than in PVH. Moreover, pretreatment with MDC markedly increased EGF-induced dimerization in PVH compared to PPH. On the other hand, retinoic acid treatment significantly reduced EGF-induced EGFR dimerization in both subpopulations (Fig. 4). Retinoic acid treatment also decreased levels of cytosolic EGFR in both subpopulations to approximately one-half of untreated cells (Fig. 5). Others have reported that retinoids reduced EGFR levels in human ectocervical epithelial cell lysates (25,26). We show here that in primary cultured rat hepatocytes, retinoic acid decreased the level of EGFR in the cytosolic fraction but not in the cell membrane fraction. The present data suggested that TG2 negatively regulated EGF-induced dimerization of EGFR. We have previously shown that TG2 regulated binding affinity of high-affinity EGFR but not that of the low-affinity receptor (13). Moreover, we have reported that calcium pretreatment induced decrease in EGF binding to EGFR through activation of TG2 in isolated

liver membranes (27). Until now, the relationship between EGFR dimerization and affinity of EGFR has not been well understood. However, it is commonly assumed that EGF preferentially binds to high-affinity EGFR, which is in equilibrium with low-affinity EGFR, and thereafter, the EGF-EGFR complexes undergo dimerization (16). Indeed, the present results show that expression of EGF-induced dimerization was better correlated with appearance of the high-affinity EGFR. Therefore, our data support the idea that binding of EGF to EGFR in high-affinity states causes dimerization of EGFR.

Recently, it was reported that monomeric EGFR formed a pre-dimer before association with EGF on the cell surface of HeLa cells (28). Pre-dimers appear to be responsible for effective formation of signalling dimers. However, in the present study, formation of EGFR dimers in the absence of EGF was almost undetectable by immunoblot analysis. Possible reasons for this disagreement are the different cell types and culture conditions.

EGF-induced EGFR dimerization promotes autophosphorylation at intracellular tyrosine residues, thereby initiating the intracellular signalling pathway (29,30) and here the pattern of phosphorylation of EGFR was nearly parallel to that of dimerization of its receptor (Figs 4 and 6). Following activation of EGFR kinase and autophosphorylation of EGFR at tyrosine 1173, activation of the mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K) are induced (31,32). This MAPK and PI3K activation is essential for EGF-induced hepatocyte proliferation in primary cultured systems. Therefore, we think that autophosphorylation of EGFR at tyrosine 1173 reflects hepatocyte proliferative capacity.

Our present data demonstrate the possibility that zonal differences in cell growth processes between PPH and PVH are at least partly caused by TG2-mediated down-regulation of EGFR dimerization, which is upstream of its phosphorylation cascade, resulting in sequential activation of MAPK or PI3K. In addition, these results are consistent with our previous report showing down-regulation of high-affinity EGFR mediated by TG2 (13). Moreover, we show here for the first time that EGFR serves as a TG2 substrate. The present data provide useful information for further studies on heterogeneity of cell growth patterns in the liver.

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