Transglutaminase differentially regulates growth signalling in rat perivenous and periportal hepatocytes

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Abstract. The influence of transglutaminase 2 (TG2) activity on the proliferative effect of epidermal growth factor (EGF) and on EGF receptor affinity in periportal hepatocytes (PPH) and perivenous hepatocytes (PVH) has been investigated using a primary culture system. PPH and PVH subpopulations have been isolated using the digitonin/collagenase perfusion technique. DNA synthesis was assessed by [^3H] thymidine incorporation into hepatocytes. The assay for binding of [^{125}I] EGF to cultured hepatocytes was analysed by Scatchard plot analysis. Pretreatment with the TG2 inhibitor monodansylcadaverine (MDC) greatly increased EGF-induced DNA synthesis in both PPH and PVH. Furthermore, [^{125}I] EGF binding studies in PVH treated with MDC indicated that high-affinity EGF receptor expression was markedly up-regulated, whereas in PPH, there was no significant effect. Treatment with retinoic acid (RA), an inducer of TG2 expression, significantly decreased EGF-induced DNA synthesis in both PPH and PVH. Binding studies in the presence of RA revealed that the high-affinity EGF receptor was down-regulated and completely absent in both PPH and PVH. These results suggest that TG2 was involved in the differential growth capacities of PPH and PVH through down-regulation of high-affinity EGF receptors.

INTRODUCTION

Epidermal growth factor (EGF) is well recognized as a potent mitogen (Herbst 2004). Trans-membrane signaling events occur when EGF binds to cell surface receptors, and a subclass of high-affinity EGF receptors contributes to activation of the EGF receptor signal transduction cascade (Defize *et al.* 1989; Bellot *et al.* 1990). Binding of EGF to the receptor activates its intrinsic tyrosine kinase and induces phosphorylation of tyrosine residues on the receptor, resulting in the induction of cell growth (Herbst 2004).

Hepatocytes of the rat liver parenchyma are classified as periportal hepatocytes (PPH) and perivenous hepatocytes (PVH) according to studies showing zonal differences in metabolism and proliferation (Jungermann 1986; Gebhardt 1988; Jungermann & Kietzmann 1996). PPH and

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PVH show different responses to EGF due, at least in part, to the heterogeneous distribution of EGF receptors (Gebhardt & Marti 1992). However, the reason for this zonal difference in the effect of EGF on cell growth in PPH and PVH is still largely unknown.

Transglutaminase 2 (TG2) is a Ca^{2+} -dependent enzyme that catalyses the post-translational modification of proteins through the formation of ϵ -(γ -glutamyl) lysine isopeptides (Fesus & Piacentini 2002; Lorand & Graham 2003). This enzyme is widely distributed in various mammalian tissues and catalyses the modification of many cellular proteins (Esposito & Caputo 2005). It has been reported that TG2 may regulate the high-affinity EGF receptor *in vitro* (Katoh *et al.* 1993). Moreover, it has recently been demonstrated that there is an inverse correlation between DNA synthesis and TG2 activity in PPH and PVH in regenerating rat liver after partial hepatectomy (Ohtake *et al.* 2004). These findings raise the possibility that TG2 affects EGF receptor binding affinity, resulting in zonal differences between cell growth in PPH and PVH through the alteration of the EGF receptor signal transduction cascade.

In the present study, to determine whether TG2 is involved in the difference in growth capacities between PPH and PVH, we investigated the influence of TG2 activity on the proliferative effect of EGF and on EGF receptor affinity in primary cultures of PPH and PVH.

MATERIALS AND METHODS

Materials

[^3H] Thymidine and [^{125}I] EGF were purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Collagenase was from Nitta Gelatin (Osaka, Japan). Digitonin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse EGF was obtained from Biomedical Technologies, Inc. (Stoughton, MA, USA). 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 at a controlled temperature ($23 \pm 1^\circ\text{C}$) under a 12-h light–dark cycle and were maintained 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

After animals were killed and livers removed, PPH and PVH subpopulations were isolated using the digitonin/collagenase perfusion technique described by Quisthoff (1985) with modifications as described by Imai *et al.* (1998). The detailed procedure has been described previously (Ohtake *et al.* 2004). After perfusion with collagenase solution, the liver lobes were carefully transferred to Dulbecco's Modified Eagle's Medium (DMEM) (Nissui, Tokyo, Japan) and were minced gently. This minced liver tissue was filtered through a 100-nylon mesh. After centrifugation at 50 g for 1 min, the supernatant was removed, and cells were resuspended in DMEM and recentrifuged. After this procedure was repeated three times, the cells were suspended in Williams E (WE) medium (Sigma-Aldrich). Viability determined by trypan blue staining was more than 90% at this point. PPH and PVH were placed in 24-well collagen-coated dishes (Iwaki, Tokyo, Japan) at a density of 3.0×10^4 cells/cm 2 or 1.0×10^5 cells/cm 2 in WE medium containing 10% fetal bovine serum (FBS), 10^{-9} M insulin, 10^{-9} M dexamethasone, 1% (v/v) antibiotics [penicillin G sodium (100 U/ml), streptomycin sulphate (100 $\mu\text{g/ml}$), amphotericin B

(0.25 µg/ml)] (Gibco, Grand Island, NY, USA). After a 3-h incubation at 37 °C in an atmosphere of 95% air and 5% CO₂ at 100% relative humidity, the medium was replaced with serum-free medium containing EGF (10⁻⁸ M), a concentration which induced the maximum proliferative effect.

Confirmation of separation of PPH and PVH

Separation of PPH and PVH was confirmed by measuring enrichment in two specific marker enzymes: alanine aminotransferase (ALT) for PPH and glutamine synthetase (GS) for PVH. ALT activity was measured by the method of Reitman and Frankel (1957), and GS activity was determined using the γ-glutamyltransferase assay as described previously (Wellner & Meister 1966).

Pretreatment of hepatocytes with MDC or RA

Hepatocytes were pretreated with MDC (0.5 mM) for 30 min. After the cells were washed three times with serum-free medium, EGF (10⁻⁸ M) was added. DNA synthesis was assayed after incubation at indicated times. EGF binding was assayed in hepatocytes pretreated with MDC after 24 h.

Hepatocytes were incubated for 3 h and then RA (5 µM) and/or EGF (10⁻⁸ M) added. DNA synthesis was assayed in the cells following incubation at indicated times. EGF binding was assayed in hepatocytes incubated with RA after 24 h.

Measurement of DNA synthesis in PPH and PVH

DNA synthesis was assessed by [³H] thymidine incorporation into hepatocytes. [³H] thymidine (0.5 µCi/ml) incorporation in PPH and PVH from 0 to 24 h, 24 to 48 h, and 48 to 72 h was measured with or without 10⁻⁸ M EGF. After incubation at indicated times, cells were washed with phosphate buffered saline (PBS) (pH 7.4), and fixed with 10% trichloroacetic acid and solubilized in 1 M NaOH. Radioactivity of [³H] 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 the incorporation [³H] thymidine expressed in dpm/mg protein. Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Assay for binding of [¹²⁵I] EGF to cultured hepatocytes

Hepatocytes were plated in 35-mm collagen-coated dishes at a density of 3.0 × 10⁴ cells/cm² or 1.0 × 10⁵ cells/cm². After attachment, hepatocytes were incubated for 24 h at 37 °C with serum-free WE medium. Culture medium was then replaced with binding buffer containing 50 mM HEPES (pH 7.4), 128 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 5 mM MgSO₄, and 0.5% BSA. Hepatocytes were then incubated for 4 h at 4 °C with binding buffer containing various concentrations of [¹²⁵I] EGF. After the incubation, hepatocytes were washed three times with ice-cold PBS and were harvested with 0.5 ml distilled water. Radioactivity of [¹²⁵I] EGF bound to hepatocytes was measured using a gamma counter (Aloca ARC-370M, Tokyo, Japan). Non-specific binding was determined in the presence of a 1000-fold concentration of unlabelled EGF and amounted to less than 10% of the total binding.

Statistical analysis

The Mann–Whitney test was used for the statistical analysis of [³H] thymidine incorporation in PPH and PVH. For the binding assay of [¹²⁵I] EGF, K_d values of PPH and PVH were compared as correlation coefficients.

Table 1. Influence of cell density on enzymatic characteristics in primary cultured PPH and PVH

Marker enzyme	Cell density	Times after primary culture (h)	PPH specific activity ^c	PVH specific activity ^c	PVH/PPH ratio
ALT ^a	high	24	425 ± 43	187 ± 26	0.44
		48	387 ± 53	218 ± 43	0.56
		72	317 ± 27	181 ± 35	0.57
	low	24	224 ± 36	172 ± 34	0.77
		48	53 ± 16	52 ± 21	0.98
		72	n.d.	n.d.	—
GS ^b	high	24	80 ± 21	956 ± 102	12
		48	83 ± 25	832 ± 131	10
		72	79 ± 18	712 ± 87	9
	low	24	67 ± 15	402 ± 23	6
		48	21 ± 8	43 ± 19	2
		72	n.d.	n.d.	—

Hepatocytes were plated at a cell density of 1.0×10^5 cells/cm² (high density) and 3.0×10^4 cells/cm² (low density). Hepatocytes were cultured in the presence of EGF for 24, 48, and 72 h. Each specific marker was measured as described in Materials and Methods. ^aSpecific activity of ALT was expressed as IU/mg protein. ^bSpecific activity of GS was expressed as mU/mg protein. ^cAll activities represent the mean ± SEM of four experiments. n.d., not detected.

RESULTS

Isolation of PPH and PVH

PPH and PVH, isolated by the digitonin/collagenase perfusion technique, were identified by measuring the activities of specific marker enzymes (ALT and GS). The activity of ALT in PPH was approximately twice that in PVH, while the activity of GS in PVH was 10 times greater than that in PPH; both of these results were consistent with previous reports (Ohtake *et al.* 2004), and in good agreement with published values (Lindros & Penttilä 1985; Gebhardt 1990), showing successful isolation of PPH and PVH.

Influence of cell density on zonal expression of specific marker enzymes in each subpopulations

Activities of specific marker enzymes (ALT and GS) in high density (1.0×10^5 cells/cm²) and low density (3.0×10^4 cells/cm²) primary cultures of PPH and PVH in the presence of EGF were measured. As shown in Table 1, PVH/PPH ratios for ALT and GS activity indicated that the characteristics of PPH and PVH were maintained when the cells were cultured at high density but not at low density. In high-density cell cultures, the PVH/PPH ratios for ALT and GS activity were consistent with previously published values (Lindros & Penttilä 1985; Gebhardt 1990). These results indicated that near-physiological conditions were achieved in primary cultures of hepatocytes at high density. Therefore, the following studies were performed using high-density cultures (1.0×10^5 cells/cm²).

EGF-induced DNA synthesis in primary cultured PPH and PVH

DNA synthesis induced by EGF treatment in primary cultured hepatocytes were investigated. Figure 1 shows the time course of DNA synthesis in PPH and PVH. In PPH, EGF-induced DNA synthesis was gradually increased to 3 times the level of control cells at 72 h. In PVH, EGF

Figure 1. EGF-induced DNA synthesis in primary cultured PPH and PVH. DNA synthesis induced by EGF (10^{-8} M) was measured by the [3 H] thymidine incorporation method as described in the Materials and Methods section. [3 H] thymidine was included from 0 to 24 h, 24 to 48 h, and 48 to 72 h. Each value represents the mean $\pm$ SEM of 3–4 samples. $\#P < 0.05$, $##P < 0.01$, versus respective EGF-untreated group.

Figure 2. Effect of TG2 inhibitor (MDC) on DNA synthesis in primary cultured PPH and PVH. Hepatocytes were pretreated with 0.5 mM MDC for 30 min. DNA synthesis induced by EGF (10^{-8} M) was measured by the [3 H] thymidine incorporation method as described in the Materials and Methods section. [3 H] thymidine was included from 0 to 24 h, 24 to 48 h, and 48 to 72 h. Each value represents the mean $\pm$ SEM of 3–4 samples. $\#P < 0.05$, $##P < 0.01$, versus EGF alone group.

increased DNA synthesis to twice the level of control cells at 72 h. The effect of EGF on DNA synthesis was greater in PPH than in PVH cultured at high density.

Effect of MDC on DNA synthesis in primary cultured PPH and PVH

Since TG2 is known to have growth-arresting properties, it was of interest to determine whether TG2 is involved in the differential growth capacities of PPH and PVH. We studied the effect of treatment with the TG2 inhibitor MDC on EGF-induced DNA synthesis in PPH and PVH (Fig. 2). In PPH, MDC treatment gradually increased EGF-induced DNA synthesis to approximately 1.5 times the level of MDC-untreated cells at 72 h. In PVH, MDC treatment greatly

Figure 3. Effect of TG2 inducer (RA) on DNA synthesis in primary cultured PPH and PVH. DNA synthesis induced by EGF (10^{-8} M) and/or RA ($5 \mu\text{M}$) was measured by the [^3H] thymidine incorporation method as described in the Materials and Methods section. [^3H] thymidine was included from 0 to 24 h, 24 to 48 h, and 48 to 72 h. Each value represents the mean $\pm$ SEM of 3–4 samples. $\#P < 0.05$, $\#\#P < 0.01$, versus EGF and RA treated group.

increased EGF-induced DNA synthesis to approximately 2.8 times the level of MDC-untreated cells at 72 h, while at 48 h, the treatment did not affect DNA synthesis. The effect of MDC on EGF-induced DNA synthesis was greater in primary cultured PVH than in PPH at 72 h.

Effect of RA on DNA synthesis in primary cultured PPH and PVH

The effect of treatment with retinoic acid (RA), a powerful inducer of TG2 expression, on EGF-induced DNA synthesis in PPH and PVH (Fig. 3) was studied. In PPH, RA treatment significantly decreased EGF-induced DNA synthesis to approximately one-half the level of RA-untreated cells at 72 h. A similar effect was obtained in PVH by RA treatment.

EGF binding to primary cultured PPH and PVH

Next, we investigated [^{125}I] EGF binding to hepatocytes. Figure 4 shows a Scatchard plot of bound/free EGF, the [^{125}I] EGF binding rate to the receptor on PPH and PVH. Figure 4 (inset) shows saturation curves of [^{125}I] EGF-specific binding to its receptor. A Scatchard plot of the binding data in PPH was curvilinear and yielded two apparent dissociation constants (K_d) of 9.01 pM (high-affinity binding sites) and 127 pM (low-affinity binding sites). The number of binding sites (B_{max}) was 0.92 (high-affinity) and 6.69 (low-affinity) pM, respectively. In PVH, K_d values were 25.7 (high-affinity) and 235 (low-affinity) pM, and B_{max} values were 1.40 (high-affinity) and 7.35 (low-affinity) pM, respectively. PPH were found to have a greater affinity than PVH for EGF, whereas there was no significant difference in B_{max} values between PPH and PVH.

Effect of MDC on the affinity of EGF receptor in primary cultured PPH and PVH

We analysed [^{125}I] EGF binding to the receptor on PPH and PVH treated with MDC (Fig. 5). Figure 5 (inset) shows saturation curves and Scatchard plot analyses of [^{125}I] EGF-specific binding to its receptor. In PPH treated with MDC, K_d values were 5.59 (high-affinity) and 165 (low-affinity) pM, and B_{max} values were 0.47 (high-affinity) and 6.76 (low-affinity) pM, respectively. On the other hand, in PVH treated with MDC, K_d values were 10.6 (high-affinity) and 375 (low-affinity)

Figure 4. Scatchard analysis of EGF binding to primary cultured PPH and PVH. After attachment, hepatocytes were incubated for 24 h at 37 °C with serum-free medium, and then incubated for 4 h at 4 °C with binding buffer containing various concentrations of [¹²⁵I] EGF. The inset panel shows saturation curves of [¹²⁵I] EGF-specific binding to its receptor. (●) PPH; (○) PVH.

Figure 5. Effect of TG2 inhibitor (MDC) on Scatchard analysis of EGF binding to primary cultured PPH and PVH. After attachment, hepatocytes were incubated for 24 h at 37 °C with serum-free medium, and were thereafter pretreated with 0.5 mM MDC for 30 min. Hepatocytes were then incubated for 4 h at 4 °C with binding buffer containing various concentrations of [¹²⁵I] EGF. The inset panel shows saturation curves of [¹²⁵I] EGF-specific binding to its receptor. (●) PPH; (○) PVH.

Figure 6. Effect of TG2 inducer (RA) on Scatchard analysis of EGF binding to primary cultured PPH and PVH. After attachment, RA (5 μ M) was added, and then hepatocytes were incubated for 24 h at 37 °C with serum-free medium. Binding assays were then performed as described in the Materials and Methods section. The inset panel shows saturation curves of [¹²⁵I] EGF-specific binding to its receptor. (●) PPH; (○) PVH.

pM, and $B_{\max}$ values were 0.72 (high-affinity) and 7.34 (low-affinity) pM, respectively. MDC treatment caused the K_d for the high-affinity receptor in PVH to reach a value similar to that of PPH.

Effect of RA on the affinity of EGF receptor in primary cultured PPH and PVH

We analysed [¹²⁵I] EGF binding to the receptor in PPH and PVH pretreated with RA (Fig. 6). Figure 6 (inset) shows saturation curves and Scatchard plot analyses of [¹²⁵I] EGF-specific binding to its receptor. The high-affinity EGF receptor was down-regulated and completely disappeared following RA treatment in both cell subpopulations. The K_d value for the low-affinity EGF receptor in PPH was 136 pM, and $B_{\max}$ was 5.87 pM. In PVH, the K_d value was 295 pM, and $B_{\max}$ was 6.19 pM. RA treatment did not affect K_d or $B_{\max}$ of the low-affinity receptor in either cell subpopulation.

DISCUSSION

In the present study, it has been demonstrated that when PPH and PVH were cultured at a high density, zonal characteristics in both subpopulations were sustained, whereas at a low density, these characteristics rapidly disappeared (Table 1). Hepatocyte proliferation and liver-specific differentiation functions are known to be reciprocally regulated by the cell density in primary culture (Nakamura *et al.* 1983a,b; Takehara *et al.* 1992). It has been reported that when hepatocytes are grown in serum-free culture at low density, they undergo a rapid loss of liver-specific

functions (Enat *et al.* 1984). Indeed, the present results have shown that specific markers in each population were abolished in cells cultured at a low density. Therefore, primary culture of hepatocytes at high density provides a good model for studying their characteristic proliferation in PPH and PVH.

It has been reported that overexpression of TG2 causes a drastic reduction in the proliferative capacity of human neuroblastoma cells (Melino *et al.* 1994). We have reported that inhibition of *de novo* synthesis of TG2 results in increased growth of normal rat hepatocytes in the presence of EGF (Katoh *et al.* 1996a) or HGF (Katoh *et al.* 1996b). Moreover, we recently demonstrated an inverse correlation between DNA synthesis and TG2 activity in PPH and PVH after partial hepatectomy (Ohtake *et al.* 2004). Also, in the present study, EGF-induced DNA synthesis was greater in PPH than in PVH (Fig. 1). Moreover, pretreatment with the TG2 inhibitor MDC greatly increased EGF-induced DNA synthesis, especially in PVH (Fig. 2). On the other hand, RA, an inducer of TG2, significantly decreased EGF-induced DNA synthesis in both cell subpopulations (Fig. 3). These results suggest that TG2 negatively regulates the EGF growth signal. We have previously reported that EGF stimulated *de novo* synthesis of TG2 in adult rat hepatocytes (Katoh *et al.* 1996a). Furthermore, we also showed that stimulation of cell growth signalling by partial hepatectomy in rats induced *de novo* synthesis of TG2 preferentially in PVH as compared with PPH (Ohtake *et al.* 2004). Considering the results of previous reports with the present ones, it is possible that TG2 tightly regulates DNA synthesis in PVH but not in PPH.

We next examined the mechanism by which these zonal differences occurred. We investigated [¹²⁵I] EGF-specific binding to the receptor on PPH and PVH treated with MDC or RA. In PPH treated with MDC, there was no significant difference in K_d and B_{max} values compared with MDC-untreated cells (Figs 4 and 5). On the other hand, in PVH, high-affinity EGF receptor expression was greatly up-regulated by treatment with MDC (Fig. 5). However, MDC did not affect K_d or B_{max} of the low-affinity receptor in either subpopulation (Fig. 5). In the case of RA treatment, the high-affinity EGF receptor was down-regulated and completely disappeared following RA treatment in both cell subpopulations (Fig. 6). RA treatment did not affect K_d or B_{max} of the low-affinity receptor in either cell subpopulation (Fig. 6). It is known that a subclass of high-affinity EGF receptors contributes to the activation of the EGF receptor signal transduction cascade (Defize *et al.* 1989; Bellot *et al.* 1990) and that only the high-affinity EGF binding site is associated with the cytoskeleton (Roy *et al.* 1991, van Bergen en Henegouwen *et al.* 1992). Previously, we have suggested that TG2 decreases the binding of EGF to the high-affinity receptor in isolated liver membranes through modification of actin (Katoh *et al.* 1993). These observations raise the possibility that newly synthesized TG2 regulates the binding affinity of the high-affinity EGF receptor but not of the low-affinity receptor, resulting in zonal differences in cell growth between PPH and PVH.

Imai *et al.* reported that PPH responded to EGF with higher sensitivity than PVH, although there was no significant difference RA treatment in EGF binding between PPH and PVH (1998). In the present study, our DNA synthesis results were consistent with those reported by Imai and associates; however, in our EGF binding study, there was a zonal difference between PPH and PVH. A possible reason for the discrepancy is the different conditions used for primary culture: Imai *et al.* cultured hepatocytes under very low cell density conditions, whereas we cultured them under high cell density conditions. In the present study, under low-density conditions, there were no zonal differences between the cell subpopulations. At this point, we could not determine the reason for indicating the zonal difference between PPH and PVH at low density.

Previous studies have indicated that primary cultured PPH and PVH show different responses to EGF, but the mechanism by which these differences occur was not clearly understood. The present data suggest that TG2 is involved in the difference in growth capacities between PPH

and PVH through down-regulation of high-affinity EGF receptors. The opinion in this laboratory is that TG2 is one of many factors involved in the regulation of DNA synthesis in hepatocytes. The present data provide valuable information for further studies on zonal differences in hepatocyte proliferation.

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