Effects of oxygen radical scavengers on connexins 32 and 26 expression in primary cultures of adult rat hepatocytes

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Although we recently reported our success in inducing and maintaining the gap junctional intercellular communication (GJIC) in rat hepatocytes cultured in serum-free medium supplemented with epidermal growth factor, dimethylsulfoxide (DMSO) and glucagon, it is known that DMSO is not only a differentiation reagent for various cells but also a powerful scavenger of oxygen radicals. In the present study, we have examined the effect of oxygen radical scavengers such as DMSO, dimethylthiourea (DMTU) and α-tocopherol on the expression of both Cxs and on gap junctional intercellular communication (GJIC), as compared to another differentiation reagent, hexamethylene-bis-acetamide (HMBA). DMSO and DMTU clearly inhibited the oxidative stress of the cultured hepatocytes, while α-tocopherol and HMBA did not. The expression of Cx26 and Cx32 in the cultured hepatocytes was markedly induced by DMSO and DMTU. Furthermore, extensive GJIC was also observed with DMSO and DMTU. These results suggest that the expression of gap junctions in the hepatocytes may be closely related to oxidative stress and that oxygen radical scavengers may be important substances in inducing this expression.

Introduction

Gap junctional intercellular communication (GJIC) is thought to play a crucial role in the maintenance of homeostasis, morphogenesis, cell differentiation and growth control in multicellular organisms (1-4). Gap junctions are intercellular membrane channels that link neighboring cells and that mediate reciprocal exchanges of molecules of <1000 daltons and ions between adjacent cells in contact (5-8), and includes second messengers such as cAMP, inositol trisphosphate and Ca\(^{2+}\). Gap junctions are composed of proteins called 'connexins' (9). In rat hepatocytes, two homologous connexin molecules have been identified (10): connexin 32 (Cx32) as a major component and 26 (Cx26) as a minor component.

We recently showed that Cx32 in primary cultures of adult rat hepatocytes is re-expressed and maintained for a long time in modified L-15 medium supplemented with epidermal growth factor (EGF) and 2% dimethylsulfoxide (DMSO) (11). Even more recently, we found that Cx26 could also be induced by the addition of glucagon together with 2% DMSO (12). The reappearing gap junctions were well maintained together with extensive GJIC. However, the mechanisms by which DMSO induces gap junctions are not yet clear.

DMSO is a dipolar, aprotic organic solvent which is active in biological systems as a cryoprotectant and a differentiating agent. For the purpose of maintaining differentiation functions, 2% DMSO is also used in primary cultures of rat hepatocytes (13-16). Isom et al. (13) first reported that adult rat hepatocytes in a chemically defined medium supplemented with EGF and 2% DMSO survived much longer and synthesized albumin much better than cells cultured in standard serum-free medium. On the other hand, it is also known that DMSO and dimethylthiourea (DMTU) are powerful scavengers of oxygen radicals (17). Recently, Villa et al. (18) reported that the property of DMSO and DMTU as scavengers of oxygen radicals played a role in the maintenance of liver-specific functions such as production of cytochrome P-450 in primary cultured rat hepatocytes.

Oxygen radical-generating compounds such as 12-O-tetradecanoylphorbol-13-acetate (TPA), 1,1-bis [4-chlorophenyl]-2,2-trichloroethane (DDT) and paraquat inhibit GJIC in various cells in culture (19-21). Sáez et al. reported that carbon tetrachloride inhibited GJIC in primary hepatocytes via the effect of oxidant stress (22). Furthermore, several antioxidants such as superoxide dismutase and α-tocopherol prevent inhibition of GJIC induced in mouse hepatocytes by the tumor promoters such as phenobarbital and DDT (23).

On the other hand, nitro blue tetrazolium (NBT) is a hydrophilic yellow crystalline substance which is converted to insoluble blue formazan by reduction (24). NBT is utilized for in vitro evaluation of macrophage or neutrophil function by measuring formazan content produced by reducing agents such as superoxide anions or NADPH oxidase (25). In primary cultures of rat hepatocytes, the NBT formazan content is increased by oxidative stress (26).

In the present study, by using our culture system and the NBT formazan assay, we have examined the effect of oxygen radical scavengers, DMSO, DMTU and α-tocopherol on the expression of both Cx26 and Cx32, and on the GJIC as compared to another differentiation reagent, hexamethylene-bis-acetamide (HMBA) (27). Our results show that the expression of these Cx genes is closely related to oxidative stress in hepatocytes.

Materials and methods

Isolation and culture of rat hepatocytes:

Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) weighing 300-400 g were used to isolate hepatocytes by the two-step liver perfusion method of Seglen (28) with some modification.
Fig. 1. Phase-contrast and the NBT formazan deposition of cultured rat hepatocytes at day 10. The medium was changed to a medium with 2% DMSO, 25 mM DMTU, 100 μM α-tocopherol and 0.5 mM HMBA from 96 h. (a,b,e,g,i): photographs of the phase-contrast; (b,d,f,h,j): photographs of NBT formazan deposition; (a,b) control; (c,d) DMSO; (e,f) DMTU; (g,h) α-tocopherol; (i,j) HMBA. Figures are the same magnification. Bars, 40 μm.

Briefly, the liver was perfused in situ through the portal vein with 150 ml of Ca²⁺, Mg²⁺-free Hank’s balanced salt solution (HBSS) supplemented with 0.5 mM EGTA (Sigma Chemical Co., St Louis, MO), 0.5 mg/l insulin (Sigma) and antibiotics. After the initial brief perfusion, the liver was perfused with 200 ml HBSS containing 40 mg of collagenase (Yakulto Co., Tokyo, Japan) for 15 min. The isolated cells were purified by Percoll iso-density centrifugation (29). Viability of the cells by the trypan blue exclusion test was >90% in these experiments. The cells were suspended in L-15 medium with 0.2% bovine serum albumin (BSA; Seikagaku Kogyo Co., Tokyo, Japan), 20 mM HEPES (Dojindo, Kumamoto, Japan), 0.5 mg/l insulin (Sigma). 10⁻⁷ M
dexamethasone (Sigma), 1 g/l galactose (Sigma), 30 mg/l proline (Sigma), and antibiotics. The isolated hepatocytes were plated on 35 mm and 60 mm culture dishes (Corning Glass Works, Corning, NY), which were coated with rat tail collagen (500 μg of dried tendon/ml of 0.1% acetic acid) (30) and placed on a 100% air incubator at 37°C. Two to three hours after plating, the medium was changed to L-15 medium supplemented with 0.2% BSA, 20 mM HEPES, 0.5 mg/ml insulin, 10^{-7} M dexamethasone, 1 g/l galactose, 30 mg/l proline, 20 mM NaHCO_{3}, 5 mg/l transferrin (Wako Pure Chemical Inc., Osaka, Japan), 0.2 mg/l CuSO_{4}-5H_{2}O, 0.5 mg/l FeSO_{4}-7H_{2}O, 0.75 mg/l ZnSO_{4}·7H_{2}O, 0.025 mg/l Na_{2}SO_{4}, 5 μg/l Na_{2}SeO_{3}, 10 μg/ml EGF (Becton Dickinson Labware, MA), and antibiotics. The cells were then placed in a humidified, 5% CO_{2}/95% air incubator at 37°C. The medium was replaced with fresh medium every other day. After each 2% DMSO (Aldrich Chemical Co., Inc., Milwaukee, WI) (11), 25 mM DMTU (Sigma) (18), 100 μM a-tocopherol (Sigma) (23) or 0.5 mM HMBA (Sigma) (27) was added to the medium supplemented with 10^{-7} M glucagon for 96 h. When viability of the cells treated for 24 h was examined by the trypan blue exclusion test, the doses of DMSO, DMTU, a-tocopherol and HMBA were found to be non-toxic.

**Measurement of the total protein and the nitro blue tetrazolium (NBT) formazan content in primary cultures of rat hepatocytes**

For measuring the total protein concentration per 35 mm dish, the cells were solubilized by phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (Sigma) and examined using a protein assay kit (Pierce Chemical Co., Rockford, IL). NBT (Sigma) was utilized for in vitro evaluation of hepatocytes by measuring formazan produced by reducing agents such as superoxide anions or NADPH oxidase (26). The cells cultured on 35 mm dishes were incubated with PBS twice. After addition of 1 ml of PBS with 2 mg/ml NBT, the cells were incubated for 30 min at 37°C. NBT was dissolved in PBS at 37°C, and filtered before use. After removal of the NBT solution, the cells were washed with 70% methanol and washed with the methanol three times. The cells were photographed, and allowed to air-dry. Formazan content in the cells was measured according to the method of Rook et al. (25) with some modifications as follows. Formazan was solubilized by adding 0.98 ml of DMSO after cell destruction with 0.84 ml of 2 M KOH. The solution was collected for measurement of formazan content at OD 630 nm. The formazan content was determined as the value per total protein.

**Immunofluorescence microscopy**

The cells grown on glass coverslips (BIOCAST, Becton Dickinson Labware) were fixed with acetone for 30 min at -20°C. After rinsing with PBS, the coverslips were incubated at room temperature (RT) for 1 h with a mouse monoclonal anti-rat Cx32 antibody (31) or a rabbit anti-rat Cx32 polyclonal antibody (32). The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse or anti-rabbit IgG (DAKO, Copenhagen, Denmark) at RT for 1 h. All samples were examined with a Nikon Fx epifluorescence photomicroscope (Nikon, Tokyo, Japan).

**Northern blot analysis**

Total RNA was extracted from the cells, using the single-step thiocyanate-phenol-chloroform extraction method (33) as modified by Xie and Robbium (34). For the electrophoresis, 10 μg of total RNAs was loaded on 1% agarose gel containing 0.5 mg/ml ethidium bromide. Gels were capillary-blotted into a nitrocellulose filter (Hybond N; Amersham Corp., Buckinghamshire, UK) and fixed by ultraviolet light. For detection of Cx32 mRNA and Cx26 mRNA, digoxigenin (DIG)-labeled RNA probes were prepared from rat Cx32 cDNAs (35) and rat Cx26 cDNAs (36) using an RNA labeling kit (Boehringer–Mannheim, Mannheim, Germany). Hybridization, washing and chemiluminescent detection were carried out according to the DIG luminescent protocol (37). Scanning-densitometry was performed using a Macintosh Quadra 800 computer (Apple Computer, Cupertino, CA) and an EPSON GT-6000 scanner (Seiko Epson, Suwa, Japan). The signals were quantified by the NIH Image 1.52 Densitometric Analysis Program (Wayne Rasband, NIH, Bethesda, MD). Expression of the transcripts was shown as a percent of 0 h (isolated hepatocytes) values in the same experiment, which was demonstrated as a histogram.

**Measurement of gap junctional intercellular communication (GJIC)**

For measuring GJIC, we used the scrape loading/dye transfer method with some modification (11). Hepatocytes on 35 mm dishes were rinsed several times with PBS. Two or three lines were made around the center of the dish using a surgical blade and 2 ml of 0.05% Lucifer yellow CH (LY; Sigma) in PBS was added to the dishes after the scrape. LY is a small molecule (457 Da) which can freely move through gap junctions from loaded cells to neighboring ones. Three minutes after the dye treatment, the cells were rinsed several times with PBS to remove excess dye. We also used rhodamine dextran (10 kDa; Sigma) which is known not to move through gap junctions, as a control dye. We immediately observed the intensity of LY transfer with

Fig. 2. The amount of the NBT formazan content per total protein in cultured rat hepatocytes. The medium was changed to the medium with 2% DMSO, 25 mM DMTU, 100 μM a-tocopherol and 0.5 mM HMBA from 96 h.

**Results**

**Phase-contrast of the hepatocytes**

The morphology of the treated cells at day 10 is shown in Figure 1. In the cells treated with 2% DMSO or 25 mM DMTU, many large cells were observed and the borders of cells were very clear (Figure 1c and e). The cells treated with 100 μM a-tocopherol or 0.5 mM HMBA did not change compared to the cells cultured in the control medium (Figure 1a, g and i).

**Effect of inhibition on oxidative stress in the hepatocytes**

To examine the effect of inhibition of oxidative stress in the cultured hepatocytes, an NBT formazan assay was carried out. When the cultured hepatocytes were incubated with NBT, NBT formazan induced by the oxidative stress was observed as a blue deposition and was monitored at OD 630 nm. The changes of NBT formazan deposition and the NBT formazan content of the hepatocytes are shown in Figures 1 and 2. In the hepatocytes cultured in the control medium, the NBT formazan content was maintained at a high level from 24 h until day 10 and the NBT formazan deposition was observed in many cells (Figure 1b). In the cells treated with 2% DMSO or 25 mM DMTU, the NBT formazan deposition was observed in some of the cells (Figure 1d and f) and the content markedly decreased at day 10. In the cells treated with 100 μM a-tocopherol or 0.5 mM HMBA, at day 10 the NBT formazan deposition and the content did not change compared to those of the cells cultured in the control medium.

**Effect on the expression of Cx26 and Cx32 in the hepatocytes**

As previously reported (12), the expression of Cx26 and 32 in hepatocytes cultured in 2% DMSO medium could be detected by immunocytochemistry and Northern blot. In this experiment, the expression of Cx26 and Cx32 of the hepatocytes treated with 25 mM DMTU, 100 μM a-tocopherol or 0.5 mM HMBA was also examined by immunocytochemistry and Northern blot, and then compared to those of the cells
Fig. 3. Fluorescence immunocytochemistry of Cx32 (a,c,e,g,l) and Cx26 (b,d,f,h,j) in cultured rat hepatocytes. The medium was changed to a medium with 2% DMSO, 25 mM DMTU, 100 μM α-tocopherol and 0.5 mM HMBA from 96 h. (a,b) 96 h after plating; (c,d,e,g,h) day 10 (6 days after treatment); (c,d) DMSO; (e,f) DMTU; (g,h) α-tocopherol; (j) HMBA. Figures are the same magnification. Bar, 10 μm.

Treated with 2% DMSO. Figure 3 shows the changes of fluorescence immunocytochemistry in the hepatocytes at 96 h and day 10 (6 days after treatment). In the cells cultured with the control medium, Cx32-positive spots were observed between adjacent cells until 72 h (11). However, after 96 h, Cx32-positive spots were rarely observed (Figure 3a). On the other hand, Cx26-positive spots were observed until 8 h (12) and thereafter they were not found in the cells (Figure 3b).
Effect of oxygen radical scavengers on gap junctions

Fig. 4. Northern blot analysis for Cx32 and Cx26 of cultured rat hepatocytes. The medium was changed to medium with 2% DMSO, 25 mM DMTU and 100 μM α-tocopherol from 96 h. (A) 0, fresh isolated cells; 96H, 96 h after plating; 6D, day 6 (2 days after treatment); 10D, day 10 (6 days after treatment); CONT, control; αT, α-tocopherol. Total RNA (10 μg/lane) was fractionated by electrophoresis in a 1% agarose-formaldehyde gel and hybridized with digoxigenin-labeled Cx26 and Cx32 cRNA probes. The lower panel of (A) shows ethidium bromide staining of ribosome RNAs before transfer to membranes. Scanning-densitometric analysis of the mRNA level was performed. Details are described in Materials and methods. The results are shown in histograms (B,C); □ DMSO, ■ DMTU, ■ α-tocopherol, ■ HMBA. Expression of the transcripts is shown as the percent of 0 h (isolated hepatocytes) values.

day 10, in the cells treated with 2% DMSO, many Cx32- and Cx26-positive spots were observed between adjacent cells (Figure 3c and d). In the cells treated with 25 mM DMTU, many Cx32-positive spots were observed just as in the cells treated with 2% DMSO, but only a few Cx32-positive spots were observed (Figure 3e and f). In the cells treated with 100 μM α-tocopherol or 0.5 mM HMBA, a few Cx32-positive spots were observed, but Cx26-positive spots were rarely observed (Figure 3g, h, i and j).

Figure 4 shows the changes in transcripts of mRNAs of Cx32 and Cx26 in the hepatocytes at day 6 and day 10. In the cells cultured with the control medium, the expression of Cx32 mRNA was gradually decreased until 96 h and then remained at a low level (11,12). The expression of Cx26 mRNA was rapidly decreased and was rarely observed after 24 h (12). In the cells treated with 2% DMSO or 25 mM DMTU from 96 h, high expression of both Cx32 and Cx26 mRNAs was observed at day 6 and 10. In the cells treated with 2% DMSO, the amount of Cx26 mRNA at day 10 was twofold that of isolated hepatocytes. In the cells treated with 25 mM DMTU, the expression of Cx32 mRNA was as high as that of the cells treated with 2% DMSO, but the expression of Cx26 mRNA was weaker than that of the cells treated with 2% DMSO. In the cells treated with 100 μM α-tocopherol or 25 mM HMBA, the expression of Cx32 mRNA was slightly observed, but that of Cx26 mRNA was rarely observed.
Effect on GJIC in the hepatocytes

To examine GJIC in the hepatocytes at day 10, scrape-loading/dye transfer was performed (Figure 5). In the cells cultured in the control medium, the dye spread was one to two cells thick (Figure 5a). In the cells treated with 2% DMSO or 25 mM DMTU, the dye reached the fifth or sixth cell from the cutting line (Figure 5b and c). In the cells treated with 100 μM α-tocopherol or 0.5 mM HMBA, the dye spread was two to three cells thick (Figure 5d and e).

Discussion

In the present study, we demonstrated that the expression of Cx26 and Cx32 of primary rat hepatocytes might be inhibited by oxidative stress and that the expression of both was induced by powerful oxygen radical scavengers such as DMSO and DMTU. Oxidative stress has been shown to inhibit GJIC in some systems (19–23,38,39). Oxygen radical-generating compounds such as TPA, DDT and paraquat inhibit GJIC in various cells in culture (19–21). Sáez et al. (22) reported that carbon tetrachloride inhibits GJIC in primary hepatocytes by the effect of oxidant stress. Several antioxidants such as superoxide dismutase and α-tocopherol prevent the inhibition of GJIC induced by phenobarbital and DDT in mouse hepatocytes (23). Furthermore, alterations in oxidative stress in neoplastic nodules may affect gap junction assembly (38,39).

It is known that DMSO and DMTU are powerful oxygen radical scavengers and that they maintain liver-specific functions in primary cultures (18). In this experiment, we observed the inhibition of oxidative stress in cultured hepatocytes by using the NBT formazan assay (26). In the hepatocytes cultured with the control medium, the NBT formazan content was maintained at a high level and the NBT formazan deposition was observed in many cells. In the cells treated with DMSO or DMTU, the NBT formazan deposition was observed in some of the cells and the content markedly decreased. We also found that DMSO and DMTU inhibited the oxidative stress of the cultured hepatocytes. On the other hand, dramatic changes in the expression of gap junctions were also observed in cells treated with DMSO or DMTU. In the cultured hepatocytes at 96 h after plating, neither Cx26- or Cx32-positive spots were observed, and the expression of both Cx26 and Cx32 mRNAs was very low. In the cells treated with DMSO or DMTU from 96 h, many Cx32-positive spots were observed between adjacent cells, and the expression of Cx32 mRNA markedly increased. In the cells treated with DMSO, as many Cx26-positive spots were observed as Cx32-positive spots and the amount of Cx26 mRNA increased to 2-fold the level of isolated hepatocytes. However, in the cells treated with DMTU, a few Cx26-positive spots were observed and the expression of Cx26 mRNA increased but was weaker than that of the cells treated with DMSO. On the other hand, in the cells treated with the weak oxygen radical scavenger α-tocopherol or the differentiation reagent HMBA, the inhibition of the oxidative stress in cultured hepatocytes were not observed. Furthermore, the expression of Cx32 mRNA was very low and fewer Cx32-positive spots were observed, while the expression of Cx26 was not detected by Northern blot analysis and immunocytochemistry. In this experiment, GJIC was also related to the expression of Cx32 in the cells as we have previously reported (12). Thus, in DMSO- or DMTU-
treated cells in which high expression of Cx32 was observed, extensive GJIC was also observed. These results suggested that because of the strong inhibition of oxidative stress, the expression of both Cx32 and Cx26, and GJIC was well induced in the cultured hepatocytes. However, the expression of Cx26, but not that of Cx32, was different among the strong oxygen radical scavengers DMSO and DMTU. This suggested that the expression of Cx26 and that of Cx32 were differentially regulated (12,40,41).

On the other hand, cytochrome P-450 of the cultured hepatocytes was also induced and maintained by the effect of the strong oxygen radical scavengers DMSO or DMTU (18). It is known that cytochrome P-450 is also sensitive to oxidative stress in hepatocytes and its maintenance is very difficult in primary cultures (18). In our experiment, the total cytochrome P-450 content of the hepatocytes treated with DMSO or DMTU was well maintained (data not shown).

Oxidative stress occurring in a variety of pathological and toxicological conditions is related to cell injury, cell death and carcinogenesis. Gap junctions are also closely related to changes in cells. In the present study, the expression of gap junctions was more sensitive to oxygen radicals. Although the expression of both Cx32 and Cx26, and GJIC was well induced in proliferated adult rat hepatocytes: use of serum-free L-15 medium supplemented with EGF and DMSO. J. Cell. Sci., 108, 1347–1357.

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