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Vitellogenin and Its mRNA Induction by Estradiol-17β in the Primary Culture of Hepatocytes in the Rainbow Trout, *Oncorhynchus mykiss*

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Vitellogenin (VTG) and VTG mRNA induction by estradiol-17β (E_2) were examined in the primary cultures of hepatocyte in the rainbow trout. Hepatocytes were precultured for 2 days, then E_2 was added and cultured for another 5 days. Media and hepatocytes were then analyzed by electrophoresis and Northern blotting for VTG and VTG mRNA, respectively. The hepatocytes were formed a few aggregates within 5 days without further spreading to a monolayer. Cell viability and high DNA content were maintained during the incubation. The hepatocyte culture with E_2 induced a weak VTG band at a molecular weight of 175 kDa on Day 2 after E_2 addition. The relative amount of VTG was expressed in percentage of total protein concentrations. VTG was gradually increased as 1.9% on Day 2, 6.3% on Day 4 and 7.3% on Day 5. VTG mRNA band was detected at about 6.6 kb in the culture with E_2 at day 1 of culture. The level of VTG mRNA expression linearly increased with time until Day 5 ($r=0.97$).

Key words: Rainbow trout, Hepatocyte culture, Estradiol-17β, Vitellogenin, Vitellogenin mRNA

Introduction

Vitellogenin (calcium-binding phospholipoglycoprotein, VTG), an egg yolk precursor protein, is synthesized in response to estrogen and is incorporated into eggs to be processed as yolk proteins in maturing females (Wallace, 1985). This protein functions as nutrients for embryo and larva development until the beginning of feeding.

In vivo VTG induction by estradiol-17β(E_2) has been reported in many teleosts (Hara and Hirai, 1978; Sundararaj and Nath, 1981; Van Bohemen et al., 1982). However, only a few studies on *in vitro* VTG induction, especially in the primary hepatocyte culture, have been published in fish (Kwon et al., 1993; Yeo and Mugiya, 1997).

A hepatocyte culture provides an excellent system to elucidate the mechanisms involved in vitellogenesis. Such studies has shown at a molecular level in avians (Mullinix et al., 1976) and amphibians (Wangh et al., 1979; Stanchfield and Yager, 1980). However, trout hepatocytes have some difficulty in attaching to dishes or substrates and in forming a monolayer. Kwon et al. (1993) succeeded in detecting VTG from the spent medium in which rainbow trout hepatocytes were cultured with E_2. Flouriot et al. (1993) reported the optimization of hepatocyte aggregate culture provided the opportunity to study VTG production and VTG mRNA expression in rainbow trout.

The present study was undertaken to establish the basic method of VTG and VTG mRNA induction in hepatocyte cultures in rainbow trout. The morphology and DNA amount of isolated hepatocytes were observed during the culture. The temporal sequence of VTG and VTG mRNA induction by E_2 were also analyzed by electrophoresis and Northern blotting, respectively.

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Material and Methods

Hepatocyte preparation and incubation

Hepatocytes were prepared following Hayashi and Ooshiro (1975) as described by Kwon et al. (1993). Cell yield and viability were determined by the Trypan Blue exclusion test (Hwang et al., 2000).

Cells were plated into a 60-mm plastic petri dish with a positive charge (Falcon) at a density of 0.3~0.8×10^6 cells/dish. William's medium E (Life Technologies, Inc.) containing 0.2 μM bovine insulin (Sigma), streptomycin (100 μg/mL), and penicillin (70 μg/mL) was used for cell culture. All incubations were carried out in 3 mL of the medium at 15℃ under 5% CO_2.

Estradiol-17β treatments

E_2 (final concentration 2×10^{-6} M; 3 μL, Sigma) in 95% ethanol was added to the dish after 2 days preincubation. The same volume (3 μL) of ethanol was added to the control cultures.

Cell morphology

Hepatocytes were precultured for 2 days, then E_2 (final conc. 2×10^{-6} M) was added to the culture, and cultured for additional 5 days. During these periods, cells were observed for morphological changes by an inverted-phase contrast microscope (Nikon).

DNA determination

DNA from cells on Days 0 to 5 after E_2 treatments was extracted by the schmidt-Thannhauser method, as modified by Munro and Fleck (1996). The amount of DNA was measured by the absorbance at 260 nm according to Wilder and Stanley (1983): DNA mg $L^{-1} = 0.0227A_{260} - 0.0018A_{232}$, where A_{260} and A_{232} are absorbance at 260 and 232 nm, respectively.

Temporal sequence of VTG and VTG mRNA induction by E_2

After a 2-day preculture, hepatocytes were incubated in the culture medium with E_2 for additional 5 days. Media and hepatocytes were collected on Days 0 to 5 after E_2 addition and VTG was analyzed by electrophoresis (SDS-PAGE) and VTG mRNA induction by Northern blotting, respectively. Control cultures were prepared by the treatments of the equivalent amount of the solvents only.

SDS-polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was described by Hwang et al. (2000). Briefly, proteins were precipitated from the media by cold trichloroacetic acid, dissolved in sample buffer (0.175 M Tris-HCl, 8 M urea, 1% SDS, and 0.5% 2-mercaptoethanol, pH 7.4), and subjected to 5~20% gradient SDS-PAGE. The gels were stained with 0.25% Coomassie brilliant blue R-250 (CBB).

Qualitative and quantitative analyses of VTG

The VTG protein was identified with the band based on the results of a previous study (Kwon et al., 1993), identified VTG band (175 kDa) as VTG (main band) by immunoblot analysis.

After SDS-PAGE, the integrated optical density (IOD) of the VTG band was measured by a Bio Image System (Millipore) and was expressed as a percentage of the IOD of the total protein including VTG.

RNA extraction

Total RNA was extracted from cultured hepatocytes using an RNA extraction kit, ISOGEN (Nippon Gene) according to the method (Chomczynski and Sacchi, 1987).

The RNA was washed in 80% ethanol, lysed with water as a total RNA sample and stored at −75℃ for Northern blot analysis.

Northern blotting

Northern blotting was described by Hwang et al. (2000). Briefly, RNA samples were mixed and applied to a 1% agarose-formaldehyde gel. Then, RNA was transferred overnight to polyvinylidene difluoride membranes (Millipore). The membranes were prehybridized with 10×SSC containing 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and 0.1 mg/mL calf thymus DNA at 65℃ for 2 h. They were hybridized overnight under the above conditions with a random-primed ^{32}P-labeled VTG probe (100,000 cpm/mL). The membranes were washed and submitted to the quantification of VTG mRNA expression by autoradiography using a Bio Image System (BAS2000; Fujix).

Results

Cell morphology

Cell morphology was investigated by the collagenase perfusion method, yielding approximately 3×10^8 hepatocytes per fish and the isolated cell viability was estimated at over 90% by the Trypan Blue exclusion test. Transfered hepatocytes firmly attached to the dish and formed small chains of 3 to 8 cells during the preculture incubation and then integrated into larger chains. As shown in Fig. 1, hepatocytes formed a few aggregates within 5 days without further spreading to a monolayer. These aggregates were maintained for at least 15 days in culture. There was no difference in cell morphology between the cultures treated with and without E_2.

Cell viability and DNA determination

Hepatocytes in the control culture showed the survival rates of 75~88% on Day 5. As shown in Fig. 2, the amount of DNA on Day 5 decreased to appoximately 85% of initial value (Day 0), whether not with treatment by E_2. These data show that hepatocytes were kept in an adequate condition during the culture.

Temporal sequence of VTG induction by E_2

After a 2-day preculture, hepatocytes were incubated with E_2 for 5 days and then media were analyzed by SDS-PAGE on Days 1, 2, 3, 4, and 5. A newly synthesized protein band identified as a main VTG band by Kwon et al. (1993). Appeared faintly at a molecular weight of 175 kDa on Day 2 and became distinct with cultured time (Fig. 3). However, the control culture without E_2 did not induce the equivalent protein upto Day 5.

As shown Fig. 4, total proteins were analyzed and the expression of VTG was gradually increased as 1.9% on Day 2, 6.3% on Day 4 and 7.3% on Day 5.

Temporal sequence of VTG mRNA induction by E_2

After a 2-day preculture, hepatocytes were cultured with E_2 and analyzed by Northern blotting.

VTG mRNA bands were detected at about 6.6 kb in cultures with E_2, while the equivalent was not induced in the culture without E_2 on Day 5 (Fig. 5). This band was detected on Day 1 and gradually increased its concentration until Day 5.

The level of VTG mRNA expression was quantified by autoradiography (Fig. 6). The expression increased with time until Day 5 (r=0.97).

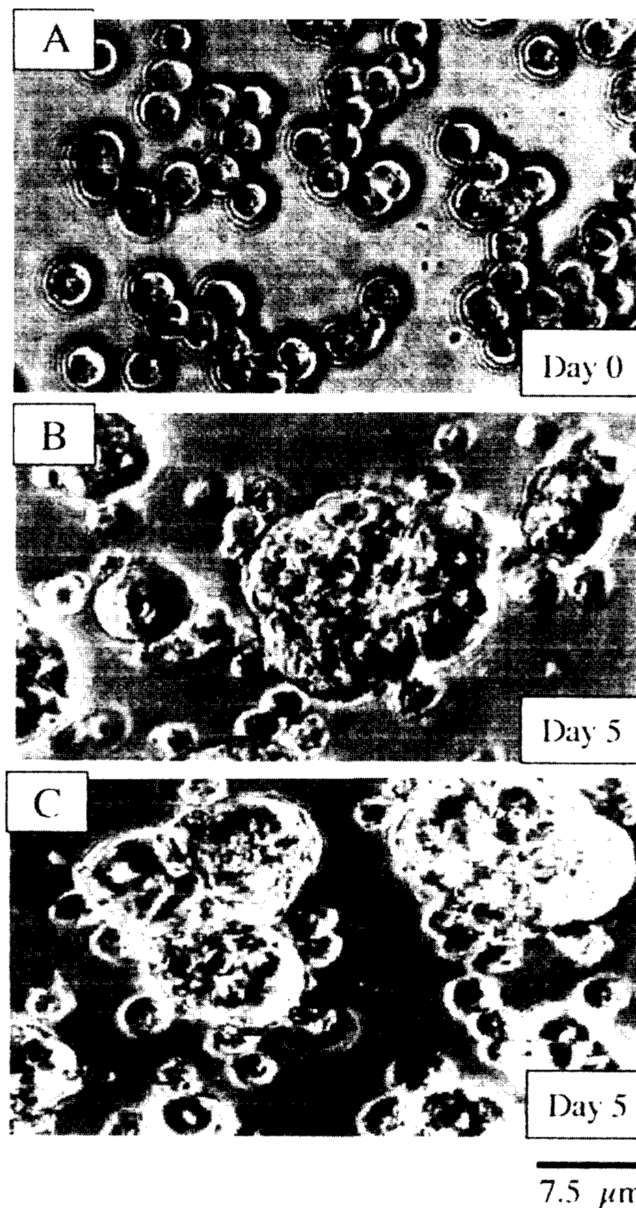

Fig. 1. Phase contrast micrographs of hepatocytes cultured in William Medium E without (A, B) and with (C) E_2 (2×10^{-6} M).

Discussion

In this experiment, VTG production consistently increased with time during at least a 5-day period

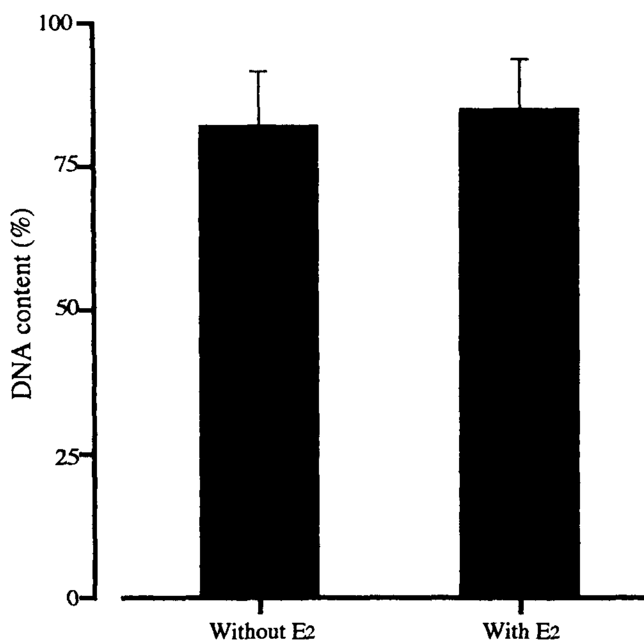

Fig. 2. Effect of E_2 (2×10^{-6} M) on hepatocyte DNA content (%) on Day 5 in culture compared with that of Day 0. Vertical bars represent SEM for triplicate.

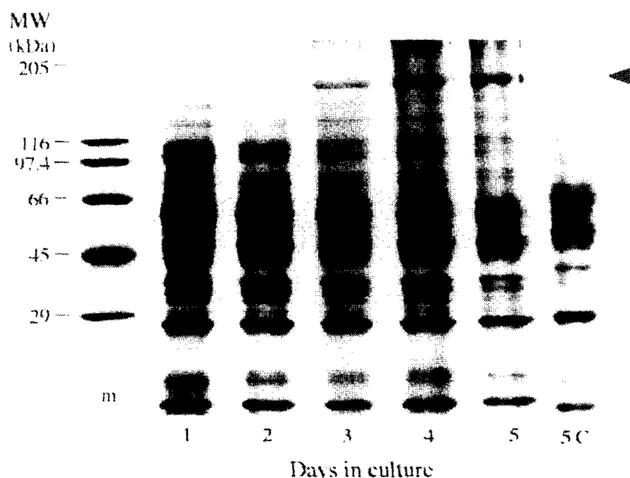

Fig. 3. SDS-PAGE showing the time-related induction of VTG (arrowhead) in hepatocyte cultures with E_2 (2×10^{-6} M) in rainbow trout. 5C: the control culture without E_2. m: molecular weight (MW) markers. Standard proteins used for molecular weight (MW) determinations were carbonic anhydrase (MW 29,000), ovalbumin (45,000), bovine serum albumin (66,000), phosphorylase *b* (97,400), β-galactosidase (116,000), and myosin (205,000). CBB stain.

after E_2 addition. We used a 5-day culture period for VTG induction, because this period was thought

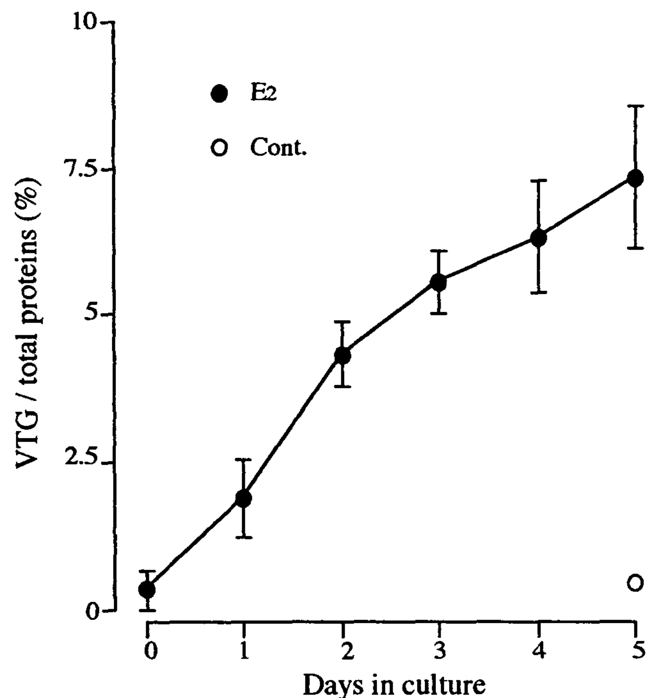

Fig. 4. Temporal sequence of VTG synthesis in hepatocyte cultures with E_2 (2×10^{-6} M). The rate of VTG synthesis was estimated for the relative optical density of VTG to total proteins after SDS-PAGE.

enough to examine the effect of E_2 on VTG production.

Maitre et al. (1986) reported that a maximum VTG production occurred on Day 3 after E_2 (10^{-6} M) addition in the hepatocyte culture of rainbow trout. Kwon et al. (1993) reported that VTG production consistently increased with time during at least a 5-day period after E_2 addition in the hepatocyte culture. Yeo and Mugiya (1997) also reported that VTG production increased at least 11 days after E_2 addition in rainbow trout.

The molecular level of vitellogenesis in fish has been become an active area of investigation in recent years. In fish, as in other oviparous vertebrates, hepatocyte culture has been used to demonstrate E_2 regulation of VTG mRNA (Vaillant et al., 1988; Flouriot et al., 1996). In this study, we also examined the effect of E_2 on VTG mRNA level. VTG mRNA level was detected at about 6.6 kb in cultures with E_2. This band was detected on Day 1 and gradually increased until Day 5. VTG mRNA level were consistently increased by E_2 during at least 5 days in culture. Mullinix et al. (1976) reported that

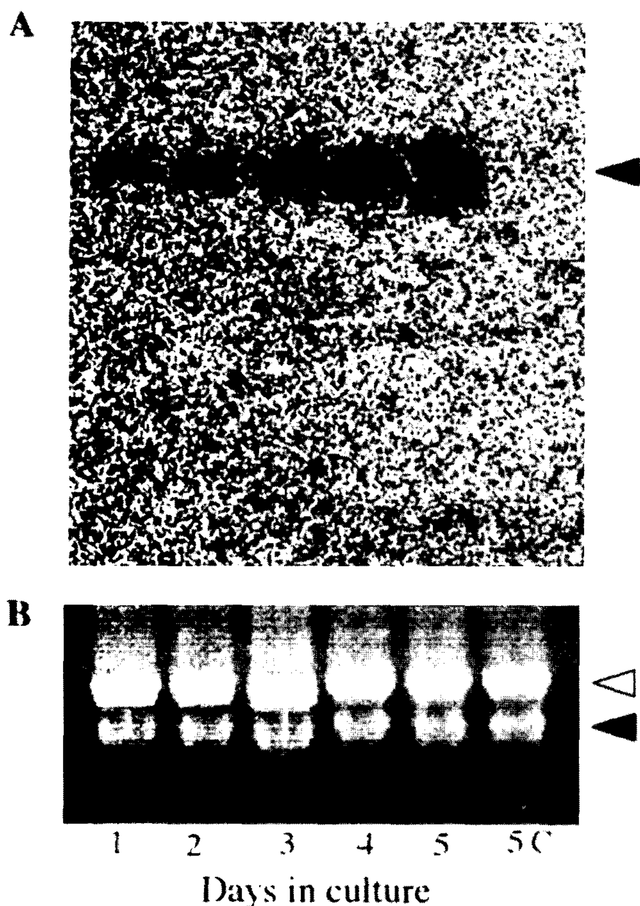

Fig. 5. Northern blotting of VTG mRNA. (A) Temporal sequence of VTG mRNA (arrowhead) expression in hepatocyte cultures with E_2 (2×10^{-6} M) in rainbow trout. Hepatocytes were extracted for total RNA on Day 5 after E_2 addition. (B) Ribosome bands with 28 S (open arrowhead) and 18 S (arrowhead). 5C: the control culture without E_2.

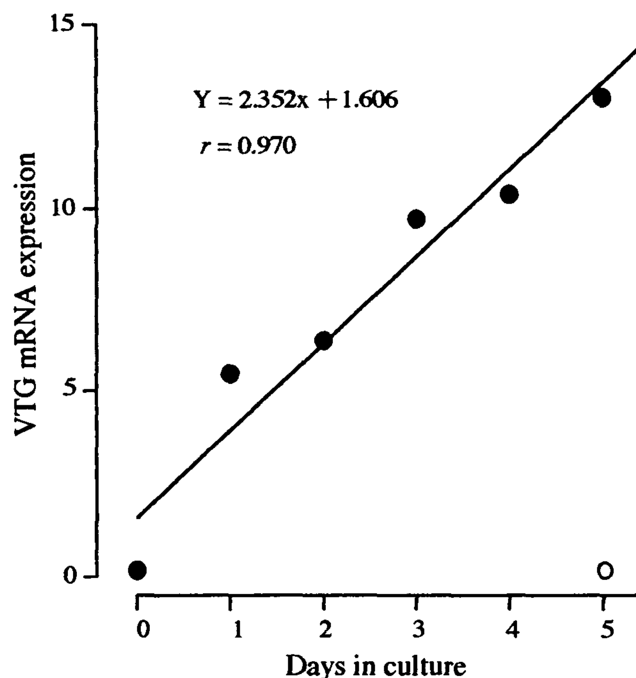

Fig. 6. Temporal sequence of VTG mRNA expression in hepatocyte cultures with E_2 (2×10^{-6} M) in rainbow trout. The activity of VTG mRNA expression was estimated by autoradiography of Northern blotting. O: control without E_2.

VTG production is largely related to VTG mRNA level in avian liver. In this experiment, VTG production closely related to VTG mRNA level and VTG mRNA level also depend on E_2 as VTG production.

Succes in hepatocyte culture closely depend on fish species. It is well known that trout hepatocytes have difficulty in attaching to a dish or substrate and in forming a monolayer (Kwon et al., 1993). In the present study, we used positively charged dishes to which trout hepatocytes attached well. Cell viability and DNA content were maintained high during incubation and much VTG and VTG mRNA synthesized were detected from the medium and hepatocytes, respectively.

Recently, ELISA (Enzyme Linked Immunosorbent Assay) has been widely used for VTG determination. In the present study, however, SDS-PAGE was used to separate VTG, before optical quantification. VTG production was expressed as a percentage of total proteins. This type of expression has the benefit of excluding the effects of variation in the number cultured cells and in the amount of proteins applied to the gel. Also, it probably has the advantage of checking the effects of hormones and toxicity on the production of proteins other than VTG. Therefore, a significant decrease in the percentage mean that the synthesis of VTG is more susceptible to added substances as hormones and toxicity than are other hepatocyte-derived proteins.

The present system of hepatocyte culture is regarded as an excellent procedure to elucidate the intracellular mechanisms whereby VTG is synthesized and also to examine effects of hormones and toxicity on Vitellogenesis.

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