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Localization of metallothionein in nuclei of growing primary cultured adult rat hepatocytes

Kazutake Tsujikawa[1], Takumi Imai[1], Makoto Kakutani[1], Yuzo Kayamori[1], Tsutomu Mimura[1], Noriko Otaki[2], Masami Kimura[2,3], Ryuichi Fukuyama[4] and Nobuyoshi Shimizu[4]

[1]*Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan,* [2]*National Institute of Industrial Health, 6-21-1 Nagao, Tama, Kawasaki, Kanagawa 211, Japan,* [3]*Central Institute for Experimental Animals, 1430 Nogawa, Miyamae, Kawasaki, Kanagawa 211, Japan* and [4]*Department of Molecular Biology, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160, Japan*

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In primary cultured adult rat hepatocytes stimulated by epidermal growth factor and insulin, dramatic changes in the subcellular distribution of metallothionein were clarified by indirect immunofluorescence using antisera specific for this protein. Metallothionein was detected only in the cytoplasm of cultured hepatocytes in the G_0 and G_1 phases, but was concentrated in the cell nuclei in the early S phase. The strongest staining pattern in the nuclei was observed 12 h after stimulation. Subsequently, the intensity of metallothionein staining in the nuclei decreased. These results suggest that primary cultured hepatocytes are suitable for examining the relation between subcellular localization of metallothionein and cell growth.

Metallothionein; Nucleus; Hepatocyte; Primary culture

1. INTRODUCTION

Metallothioneins (MTs), low molecular-weight, heavy-metal-binding, cysteine-rich cytosolic proteins were first isolated from horse kidney and characterized by Vallee and co-workers [1,2]. Since then, MTs have been found in a number of species [3]. The function of MT has been debated ever since its discovery. A role in metal metabolism or detoxification is strongly suggested by the ability of MTs to bind to and be induced by heavy metal ions [4]. Kelley et al. reported that overexpression of MT represents one mechanism of resistance to anticancer agents [5]. In recent years, immunohistochemical methods have provided new interesting information on MT intracellular distribution in the hepatocytes. MT is mainly localized in the nuclei of both fetal and neonatal rat hepatocytes, and the intranuclear localization of MT decreases with age [6]. In human liver, the localization of MT is very similar to that in rat hepatocytes [7]. However, the biological significance of the presence of MT in nuclei is not yet clearly understood.

We observed that MT suppressed the formation of gastric ulcer in rats [8]. Moreover, immunohistochemical studies showed that MT was localized in the nuclei of growing primary cultured rat gastric mucosal cells [9]. We assumed that investigation of the function of MT in nuclei was essential to determining the physiological roles played by MT. The present study was designed to examine the subcellular localization of MT in primary cultured adult rat hepatocytes stimulated by epidermal growth factor (EGF) and insulin.

Correspondence address: Kazutake Tsujikawa, Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan. Fax: (81) (6) 877 7095

Abbreviations: EGF, epidermal growth factor; MT, metallothionein

2. MATERIALS AND METHODS

2.1. *Anti-MT antibody*

Female Japanese White rabbits were initially injected subcutaneously with 1 mg of Cd MT-II, which was purified from Cd-administered rat liver, mixed 1:1 with complete Freund adjuvant. After the first injection, animals were boosted every 4 weeks 5 times each with 1 mg of Cd MT-II diluted with an equal volume of incomplete Freund adjuvant and bled 7 days after the last injection.

2.2. *Primary culture of hepatocytes*

Parenchymal hepatocytes were isolated from male Wistar rats weighing 160–200 g by in situ perfusion of the liver with collagenase [10]. The isolated cells were suspended at 1.5×10^5 cells per ml in Williams E medium containing 5% calf serum and 1 nM dexamethasone. Viability by Trypan blue exclusion test was more than 80%. Hepatocytes were inoculated at 1.5×10^5 cells per 3 cm plastic culture dish, and were cultured in the medium at 37°C under 5% CO_2. After culture for 6 h, the medium was changed to hormone- and serum-free Williams E medium and incubation was continued for 22 h before addition of EGF and insulin.

2.3. *Indirect immunofluorescence*

The intracellular localization of MT was determined by indirect im-

munofluorescence using rabbit anti-MT polyclonal antibody. At the indicated times, cultured hepatocytes were washed with phosphate-buffered saline, fixed and permeabilized by exposure to methanol at −20°C for 2 min, and incubated with anti-MT antibody (1 : 500 dilution) followed by fluorescein-conjugated goat anti-rabbit antibody (1 : 100 dilution). The stained cells were observed with a Bio-Rad laser confocal scanning microscope MRC-600 [11].

2.4. *Assay of DNA synthesis*

[^{3}H]Thymidine incorporation into hepatocytes was assayed by the method of Nakamura et al. [12].

3. RESULTS AND DISCUSSION

In determining the physiological roles of MT, it is necessary to ascertain its function not only in the cell cytoplasm but also in the nucleus. Therefore, we attempted to establish an ideal model system for examining the translocation of MT in vitro, that would give results identical to what has been observed in vivo. First, we raised a highly specific antiserum against rat

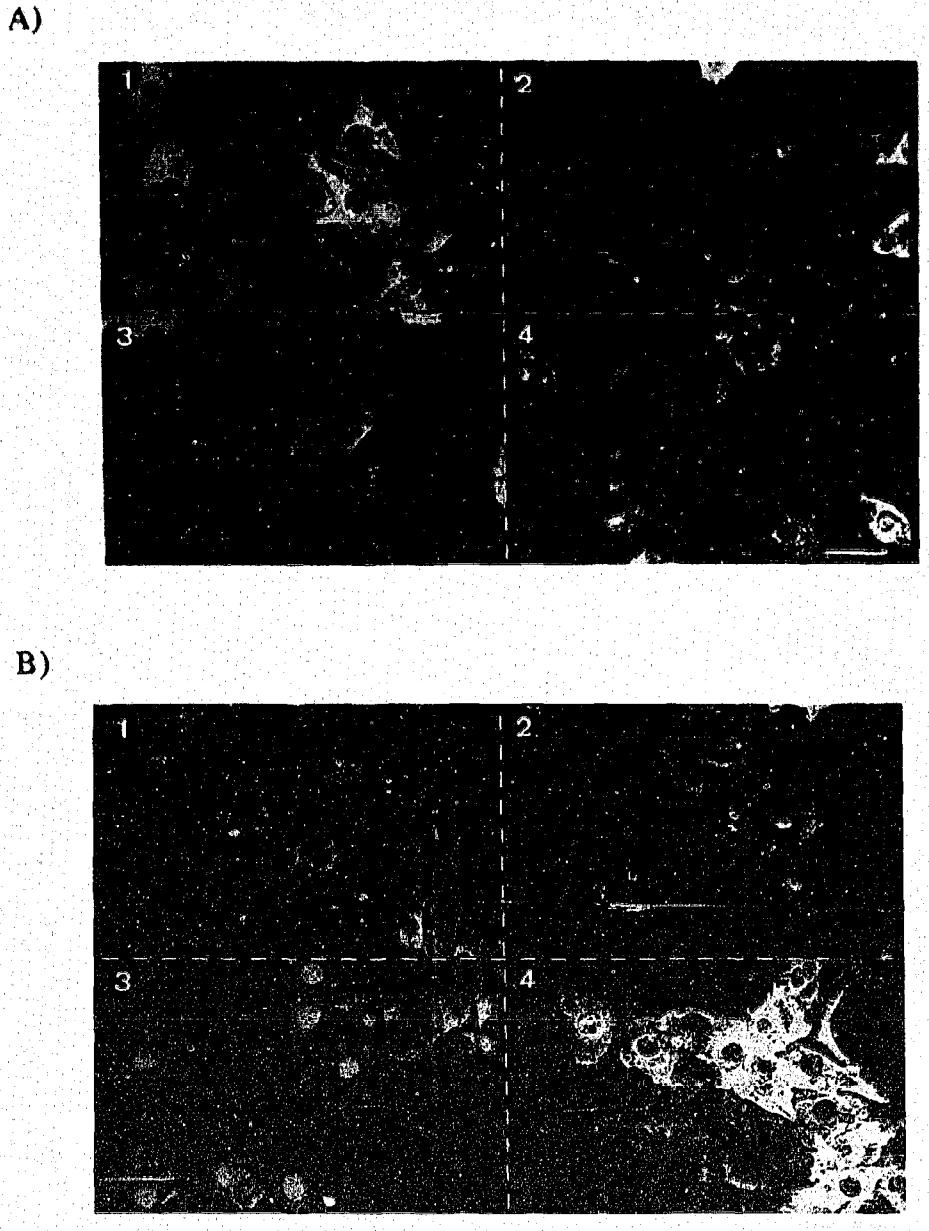

Fig. 1. MT staining patterns in adult rat hepatocytes cultured in the presence or absence of EGF and insulin. Hepatocytes were cultured on glass coverslips for 22 h and then the medium was replaced by fresh medium with or without 0.1 μM insulin and 20 ng/ml EGF. The cells were fixed and stained using anti-MT antibody and fluorescein-conjugated anti-rabbit antibody (1) 6 h, (2) 9 h, (3) 12 h, and (4) 24 h after medium change. (A) No addition; (B) with insulin and EGF. The staining signal with normal rabbit serum was substracted as a control by image processing. Bar = 50 μm.

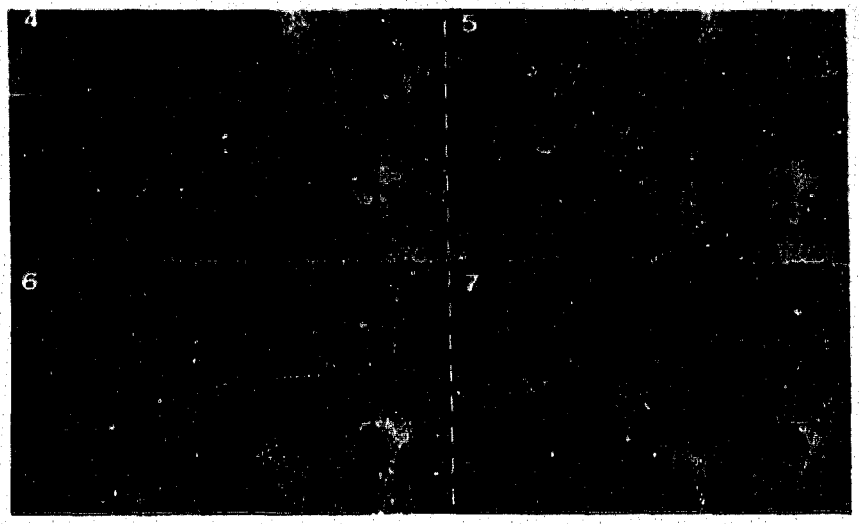

Fig. 2. Fluorescent images of primary cultured rat hepatocytes stained with anti-MT antibody. Hepatocytes were stained with anti-MT antibody 12 h after addition of 20 ng/ml EGF and 0.1 μM insulin. Fluorescent images at every 1 μm between 4 μm (4), and 7 μm (7) from the cell base are present. The staining signal with normal rabbit serum was substracted as a control by image processing.

liver MT in rabbit. Our polyclonal rabbit antibody to rat liver Cd-MT-II cross-reacted equally with both the isoforms of MT (MT-I and MT-II) from rat liver (data not shown). Second, this anti-MT antibody was used immunohistochemically to examine the localization of MT in the primary cultured rat hepatocytes. In primary culture, adult rat hepatocytes in G_0 of the cell cycle are stimulated by EGF and insulin to enter into the S phase (from G_0/G_1) [12]. MT staining patterns of cultured hepatocytes stimulated by EGF and insulin are presented in Fig. 1B. MT staining was confined to the cytoplasm 6 h after stimulation. Nine hours after stimulation, MT was localized in the nuclei. The strongest staining pattern of MT in nuclei (with exception of the nucleoli) was observed 12 h after stimulation. The absence of nucleolar staining was clearly demonstrated by tomography (Fig. 2). The reason for the absence of nucleolar MT staining is unclear. MT staining in nuclei appeared to be weaker than in the cytoplasm 24 h after stimulation. Since no MT-positive nuclei were observed in control hepatocytes in G_0 and G_1 phases (Fig. 1A) and the absorption of rabbit anti-MT with rat liver MT abolished the staining in nuclei (data not shown), the stimulation of proliferation by EGF and insulin may be necessary for localization of MT in nuclei of primary cultured adult rat hepatocytes.

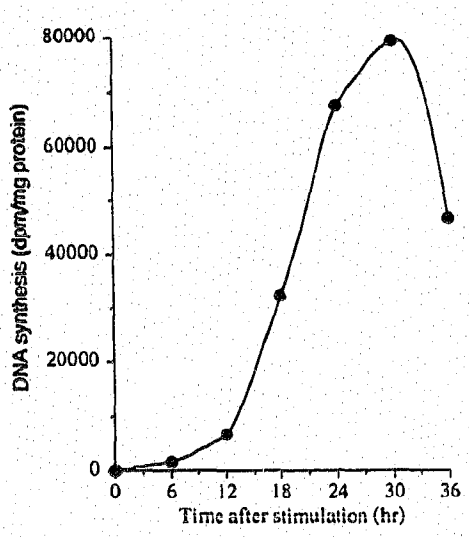

Fig. 3. Time course of DNA synthesis in primary cultured rat hepatocytes treated with EGF and insulin. EGF (20 ng/ml) and insulin (1×10^{-7} M) were added during culture and [^{3}H]thymidine was added 2 h before assay of DNA synthesis.

The position of the different subcellular localizations of MT in the cell cycle was determined by [^{3}H]-thymidine incorporation in the cultured hepatocytes (Fig. 3). In primary cultured adult rat hepatocytes, DNA synthesis started between 6 and 12 h, and was maximal 30 h after addition of EGF and insulin. These results suggested that MT detected in the cytoplasm of the hepatocytes in the G_1 phase was localized in the cell nuclei in the early S phase. Moreover, we found MT staining in nuclei of primary cultured remnant rat hepatocytes following 70% removal of the liver (data not shown). Francavilla et al. reported that remnant hepatocytes are committed to synthesize DNA very early after partial hepatectomy [13]. We suggest that the change in localization of MT is related to cell proliferation, especially to stages of the cell cycle.

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