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Metallothionein Induction in Liver Regeneration Stimulated by Partial Hepatectomy

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Metallothionein (MT) is induced in the regenerating rat liver. We have investigated expression of MT gene by RT PCR as well as specific localization of MT by immunocytochemistry in regenerating rat liver after partial hepatectomy (PH). MT mRNA level started to increase from 1 h and reached the peak at 8 h after PH. The level decreased gradually by 24 h, and became similar to that of control group. In the immunocytochemical study, in all groups treated with primary antibody, immunogold particles indicating the presence of MT were evenly distributed throughout both cytoplasm and nucleus of the rat hepatocytes. Within the nucleus, the gold particles appeared to be intensely localized in the areas of euchromatin and nucleolus. Within the cytoplasm, gold particles did not seem to adhere to mitochondria or lysosomes, but were freely distributed. However, rough endoplasmic reticulum was the obvious compartment on which the gold particles were localized. Time course of MT immunoreactivity revealed that distribution of gold particles in hepatocytes increased gradually by 24 h, and decreased at 48 h after PH. Briefly, PH resulted in the sharpest increase in the expression of MT mRNA at 8 h and in the immunoreactivity of MT at 24 h, respectively. It is suggested that the increase of MT mRNA expression, the intensity of immunoreactivity and the specific localization of MT may be associated with the compensatory cell proliferation followed by PH.

Metallothionein (MT) was first isolated from equine kidney cortex (Margoshes and Vallee, 1957). MT is a family of ubiquitous, low molecular weight (6000-7000), cysteine-rich (30-35%) inducible protein with a high affinity to metal ions and has no aromatic amino acids or histidine. Some of the known functions of MT include detoxification of heavy metals and alkylating agents and neutralization of free radicals (Hamer, 1986; Kagi and Kojima, 1987; Palmiter, 1998). Because MT isolated under physiological conditions from many species including human contains zinc, cadmium and sometimes copper, it is likely that MT plays an important role in the absorption, transport, and metabolism of these essential heavy metals. Nonetheless, the definitive physiological functions of MT are still unknown (Simpkins, 2000).

On the other hand, MT expression is related to zinc accumulation in certain organs. Evidence has been produced, which suggests that MT could act in a number of biochemical processes. It may act in zinc trafficking and/or zinc donation to apoproteins, including zinc finger proteins that act in cellular signaling and transcriptional regulation. As a result, MT expression may affect a number of cellular processes including gene expression, apoptosis, proliferation and differentiation (Park et al., 1998; Davis and Cousins, 2000; Nguyen et al., 2000). In human, MTs are encoded by a family of genes located at chromosome 16q13, containing ten functional and seven non-functional isoforms. Recently, reverse transcriptase polymerase chain reaction (RT-PCR) analysis demonstrated expression of MT in normal kidney (Mididoddi et al., 1996) and it has been reported that biosynthesis of various signals including MT was induced in the partially hepatectomized rat liver (Margeli et al., 1994; Xu et al., 2000). Many reports on the physiological and biochemical properties of MT have been published, but ultrastructural reports on the localization of MT using hepatectomized rat liver are extremely rare (Leyshon-Sorland and Stang, 1993). Using histochemical techniques, we obtained a preliminary indication that MT was induced by PH.

In recent years, application of the tools of cell and molecular biology has allowed rapid advancement in knowledge of the mechanisms underlying liver regeneration after partial hepatectomy (PH), but the exact factors or signals that stimulate and modulate liver regeneration are still not clear. After 70% of rat liver is surgically removed, the remaining hepatic cells rapidly resume cell cycle.

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The present study was undertaken to examine the time course of MT mRNA and protein induction in the process of liver regeneration stimulated by PH. We cairried out RT PCR to determine MT gene expression and immunogold labelling to localize the subcellular distributions of MT in regenerating rat liver.

Materials and Methods

Materials and hepatectomy

For partial hepatectomy (PH), male Sprague-Dawley rats (200 g) were used. The left lateral and median lobes of the liver (approximately 70% of total liver weight) were removed from the rats which were under light ethyl ether anesthesia (Waynforth, 1980). The liver remnants were taken out at specified times from 1 to 48 h after the operation for further analysis. Sham surgery was performed by subjecting rats to midventral laparotomy and closure. The IgG monoclonal antibody (Sigma), raised using horse MT-1 and MT-2 as the immunogen, is specifically reactive with a conserved epitope common to several mammalian species of MT.

MT mRNA expression analysis

Total RNA was extracted from the rat liver tissues by homogenation in guanidine isothiocyanate, followed by centrifugation. Extracted total RNA was quantified by absorbance measurements at 260 and 280 nm and stored at -80℃. PCR quantification of MT mRNA (1 μg) was performed by using gene-specific primers and ethidium bromide. The sequences of the oligonucleotide primers used for rat MT were 5'-ACTGCCTTCTT-GTCGCTTA-3' and 5'-TGGAGGTGTACGGCAAGACT-3', sense and antisense, respectively (Suzuki et al., 1998). They spanned 310 bp fragment. MT mRNA levels were corrected for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of sense and antisense primers for GAPDH were 5'-AATGCATCCTGCACCACCAA-3' and 5'-GTAGCCA-TATTCATTGTCATA-3', respectively.

Electron microscopic immunogold procedure for metallothionein(MT)

Liver tissues were cut into pieces (1 mm^3) and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4, containing 0.1% $CaCl_2$) for 4 h. After rinsing in sodium phosphate buffer, the samples were dehydrated with ethanol and embeded in araldite resin. The ultrathin sections were cut on a ultramicrotome and collected on nickel grids. The ultrathin sections were incubated with 10% H_2O_2, 10% gelatin and 0.01 M glycine solutions for 10 min each to block nonspecific reactions. They were treated with phosphate buffered saline (PBS, containing 1% goat serum) for 1 h. And then they were incubated in the presence of diluted monoclonal antibody to MT (× 50) at 4℃ in humidified

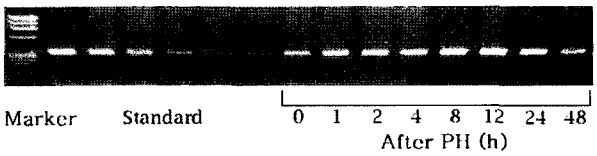

Fig. 1. Changes in MT mRNA levels with time after PH analyzed by RT PCR. MT mRNA was significantly up-regulated at 8 h after PH. Marker: 1,358, 1078, 872, 603, 310, 281 bp ; standard: 4, 2, 1, 0.5, 0.25, 0.125 μg/10 μl, respectively.

chamber for 4 h (Zhou and Kang, 2000). The degree of non-specific labelling was checked in the present study by omission of primary antiserum (anti-MT). After rinsing in PBS (containing 1% goat serum) for 5 min four times, the ultrathin sections were incubated in 10 nm gold-conjugated goat anti-mouse IgG diluted (× 200) in PBS for 4 h, and then they were rinsed in PBS and distilled water. They were stained with uranyl acetate. The labeled ultrathin sections were observed with a JEOL transmission electron microscope.

Results

Gene expression of MT after PH

The amount of RNA applied to each lane of the gel was standardized by spectrophotometric determination and judged to be similar by analysis with a GAPDH probe. The time course of expression pattern of MT after PH was compared with that of sham control. MT mRNA level of rat liver started to increase from 1 h and reached the peak at 8 h. The level decreased gradually by 24 h, and was similar to that of control group at 48 h after PH (Fig. 1).

Immunocytochemistry of metallothionein

Under transmission electron microscopy, no gold particles were found in the liver tissue sections of groups treated with pre-immune serum instead of primary antibody, as a experimental control. In all groups treated with primary antibody, gold particles indicating the presence of MT were more or less distributed throughout the cytoplasm and nucleus of rat hepatocytes, with the labelling slightly stronger in the nucleus. In experimental groups, the gold particles increased with time after PH. Especially at 24 h after PH, the immunogold labelling was significantly increased. Within the nucleus, the gold particles appeared to be intensely distributed in the areas of euchromatin and the nucleolus, while few gold particles were observed in the region of electron-dense heterochromatin. Meanwhile, within the cytoplasm, gold particles did not seem to adhere to any membranous structures such as mitochondria, Golgi complex, or lysosomes, specifically, but were freely distributed. However, the particles were often localized near the rough endoplasmic reticulum. Time course of MT immunoreactivity revealed that distribution of gold particles in hepatocytes showed the

highest level of MT immunoreactivity at 24 h, and then decreased at 48 h (Fig. 2).

Discussion

Liver regeneration is a fundamental parameter of liver response to injury. This regeneration process is an orchestrated response induced by specific external stimuli involving sequential changes in gene expression, growth factor production, and morphological structure (Matsumoto and Nakamura, 1998; Michalopoulos and DeFrance, 1998). Partial hepatectomy (PH) is the most often used stimulus to study liver regeneration because, compared with other methods that use hepatic toxins, it is not associated with tissue injury and inflammation, and the initiation of the regenerative stimulus is precisely defined. In this experiment, when two-thirds of the rat liver was removed and the remnant lobes remained intact, the residual lobes enlarged to make up for the mass of the removed lobes, though the resected lobes never grew back. We found that the remnant liver was completely restored within 5 d posthepatectomy.

Despite many studies that have been carried out on

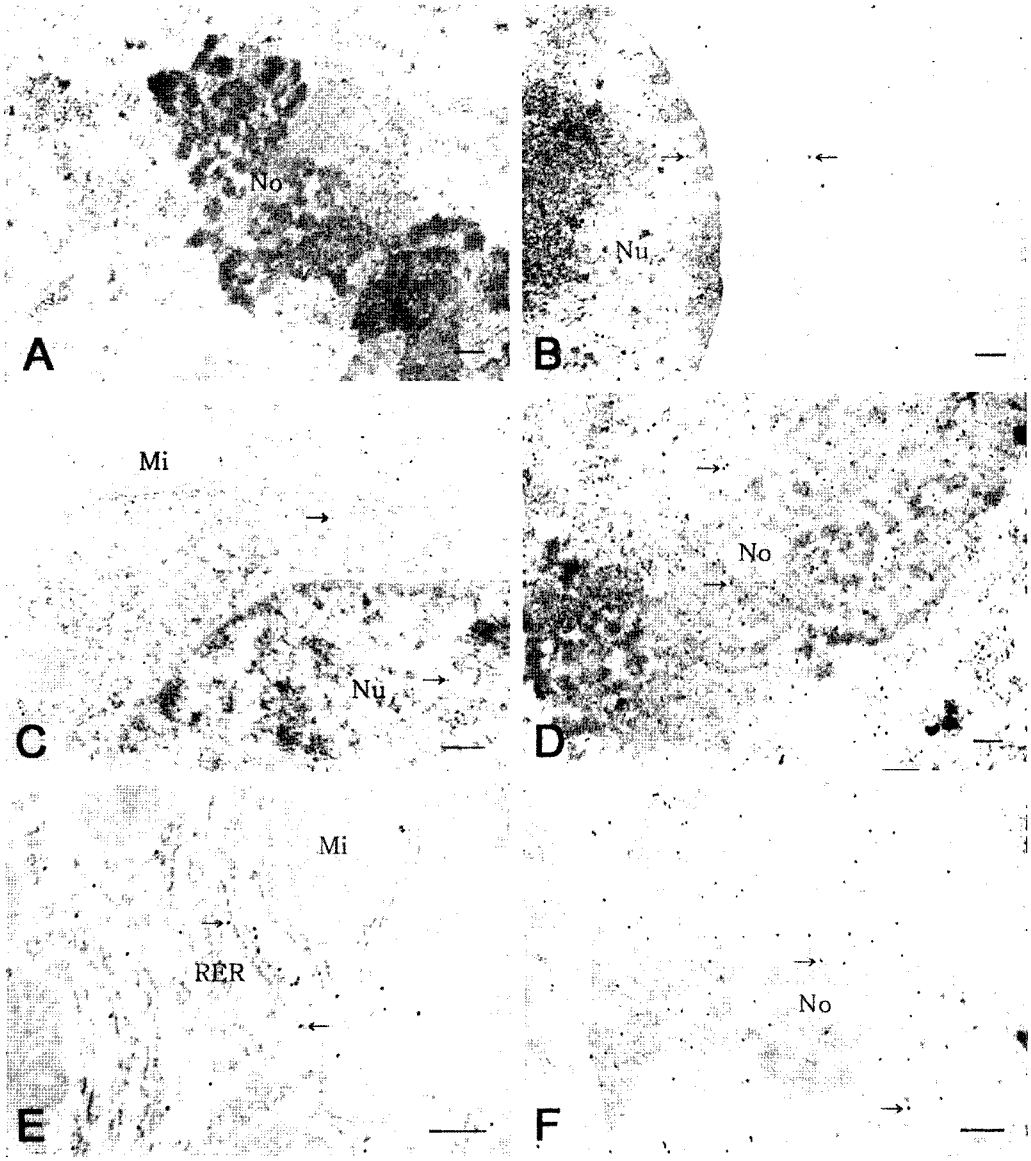

Fig. 2. Electron micrographs of immunogold labelling (arrow) of MT in the rat hepatocyte. Negative labellings for MT display in the nucleus in control (without primary anti-MT) at 24 h after PH (A). In hepatocyte of sham control rat, gold particles indicating the labelling for MT are more distributed in the nucleus than in the cytoplasm (B). This pattern of gold labelling is similarly seen in rat hepatocyte nucleus at 1 h after PH (C). Increased gold particles are ultrastructurally observed in hepatocyte nucleus, particularly adjacent to nucleolus of rat at 24 h after PH (D). The particles are localized near the rough endoplasmic reticulum in the cytoplasm of rat at 24 h after PH (E). At 48 h after PH, the gold particles is fairly distributed in hepatocyte of rat (F). Mi; mitochondrion, No; nucleolus, Nu; nucleus, RER; rough endoplasmic reticulum. Scale bars = 200 nm.

metallothionein (MT) during the past 40 yr, the primary function of the protein remains unknown. It is likely that no single function will be seen to occupy a central position in cell physiology (Naganuma, 1997). Although various factors cause a predominant localization of MT in the nucleus during the phase, it is not clear whether MT exists inside, or merely adjacent to, the nuclear compartment. Moreover, very little is known concerning the ultrastructural localization of MT in liver tissue. In this study, we have investigated the expression of MT mRNA by RT PCR and the ultrastructural localization of MT by immnogold labelling in the remnant liver after PH in rat.

The primary goal of this study was to determine MT mRNA levels and immunogold labelling in rat liver with time after PH. Experimental evidence has shown the site of MT synthesis in most tissues to be the free polyribosomes, strongly implying that the function of MT is limited to intracellular processes. The nearly complete lack of mitochondrial labelling was common in all groups and these results were consistent with previous reports (Tohyama et al., 1993; Tsujikawa et al., 1994).

It is evident from the immunocytochemical data of the present study that MT should not be viewed as an exclusively cytoplasmic protein. Probably most important is the localization of MT within the nucleus. The increased labelling of gold particles in the nucleus of the regenerating liver cell suggests that MT could be transferred from the cytoplasm to the nucleus in response to the stimulus of PH. The possibility is that MT may play a metallo-regulatory role in cell proliferation. Tsujikawa et al. (1994) reported that MT was induced and was translocated into the nuclei from the cytoplasm of hepatocytes in the remnant liver after 70% removal. As mentioned above, in the remnant liver after PH, induction of MT mRNA was preceded by an increase in the MT protein (Ghoshal and Jacob, 2000). It has been proposed that in proliferating cells, MT may act as a storage protein for zinc which is required for certain enzymes in replication and transcription factors (Davis and Cousins, 2000).

In conclusion, the increase of MT mRNA expression, the intensity of immunoreactivity and the specific localization of MT may be associated with the compensatory cell proliferation followed by PH. Further studies are needed to evaluate the precise mechanism of MT gene induction and its physiological significance in regenerating liver.

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