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Immunogold Labellings and Expression of Metallothionein in Regenerating Rat Liver

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재생중인 흰쥐 간의 메탈로사이오닌에 대한 면역-금 표지 및 발현에 관한 연구

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ABSTRACT

Metallothionein (MT) is a low molecular weight, cysteine rich, metal binding, and non enzymatic protein. The present study was carried out to investigate the expression of MT gene as well as the localization of MT in regenerating rat liver. In partial hepatectomized rats, MT mRNA was detected as early as 1 hr and reached a maximal level by 8 hr after the operation. Thereafter, this level decreased gradually until 24 hr, and it became similar to that of sham control. Meanwhile, time course of MT immunoreactivity using immunogold labelling revealed that the number of gold particles in hepatocytes increased significantly by 12 hr, but decreased at 48 hr after partial hepatectomy. Ultrastructurally, immunogold particles indicating the presence of MT were distributed in both the cytoplasm and the nucleus of the rat hepatocytes, particularly the particles were distributed at rough endoplasmic reticulum and nucleolus and did not seem to adhere to mitochondria or lysosomes in proliferating hepatocytes. Briefly, high level of MT mRNA expression and the intense immunoreactivity and/or the specific localization of MT was observed during liver regeneration. These results suggest that MT possibly involves hepatocyte proliferation via the storage or the supply of various metal ions in the regenerating rat liver.

Key words : Immunocytochemistry, Metallothionein, Partial hepatectomy, Rat

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INTRODUCTION

Metallothionein (MT) was first isolated from equine kidney cortex (Margoshes & Vallee, 1957). MT is a single-chain protein which is a molecular weight of 6000~7000 Da, usually containing 61 amino acid residues, among them 20 cysteines and binding a total of 7 equivalents of bivalent metal ions. There are four known isoforms of MT, namely MT-I, MT-II, MT-III and MT-IV. MT-I and MT-II isoforms have a ubiquitous tissue distribution and expression with particular abundance in liver, pancreas, intestine and kidney. MT-III is expressed mainly in the brain and MT-IV in squamous epithelia (Suzuki et al., 1998). MT is induced by external stimuli in various physiological conditions, chemical agents, heavy metals, stress and partial hepatectomy (PH) (Margeli et al., 1994: Kim & Shin, 2001).

Some of the known functions of MT include detoxification of heavy metals and alkylating agents and neutralization of free radicals (Hamer, 1986; Kagi & Kojima, 1987; Palmiter, 1998). Because MT isolated under physiological conditions from many species including human beings 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). And also, 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 & Cousins, 2000; Nguyen et al., 2000). In humans, the 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 regenerating rat liver are rare (Leyshon-Sorland & Stang, 1993; Oh et al., 2004). MT has been documented as being induced in various cells by a wide range of inducers, such as heavy metals, hormones, cytokines and stress stimuli. So, liver regeneration after PH is likely influenced by changes in such proteins.

On the other hand, liver performs many vital functions in the body such as metabolism of nutrients, detoxification of harmful substances, and excretion of bile. 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 (Taub, 1996).

We have undertaken to examine the induction of MT mRNA and MT protein in the process of regeneration of rat liver stimulated by PH. So, we cairried out RT-PCR to determine the MT gene expression and immunogold labelling to localize subcellular distributions of MT during the regeneration of rat liver.

MATERIALS AND METHODS

1. Animals and hepatectomy

Sprague-Dowley rats (200 g) were used in this experiment. For partial hepatectomy, 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 hr to 8 days after the operation for further analysis. Sham surgery was performed by subjecting rats to midventral laparotomy and closure. On the specific days during regeneration rats were sacrificed by decapitation and livers were excised. The IgG monoclonal antibody (Sigma Co.) used in the experiment, 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.

2. 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°C. 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′-ACTGCCTTCTTGTCGCTTA-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 sequenses of sense and antisense primers for GAPDH were 5′-AATGCATCCTGCACCACCAA-3′ and 5′-GTAGCCATATTCATTGTCATA-3′ respectively. 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.

3. Electron microscopic immunogold labelling 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 hours. 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 sections were incubated with 10% H_2O_2, 10% gelatin and 0.01 M glycine solutions for 10 minutes for each to block nonspecific reactions. They were treated with phosphate buffered saline (PBS, containing 1% goat serum) for 1 hour. And then they were incubated in the presence of diluted monoclonal antibody to MT (×50) at 4°C in humidified chamber for 4 hours (Zhou & Kang, 2000; Kim & Shin, 2001). 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 minutes four times, the ultrathin sections were incubated in 10 nm gold-conjugated goat anti-mouse IgG diluted (×200) in PBS for 4 hours, 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 AND DISCUSSION

The primary goal of this study to determine the MT mRNA levels and the immunogold labelling of MT in rat liver during regeneration after PH. The expression pattern of metallothionein (MT) mRNA with time courses after partial hepatectomy (PH) was compared with that of sham control. MT mRNA level of rat liver started to increase from 1 hr and reached to the peak at 8 hr after PH. The level decreased gradually by 24 hr, and then the pattern was similar to that of control group at 48 hr after PH (Fig. 1).

Under transmission electron microscopy, ultrastructural alterations during liver regeneration were characterized by the high nuclear-cytoplasmic ratio and prominent nuleoli, numerous small mitochondria, well developed rough endoplasmic reticulum and decreased glycogen granules within the cytoplasm (Figs. 2-5). These results could be thought as properties of active

proliferating cells.

By immunocytochemical analysis, 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 (Fig. 6). 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 (Fig. 7). Compared the reaction intensities, the labelling was slightly stronger in the nucleus than in the cytoplasm at early stage of regeneration. In experimental groups, the gold particles became increased with times after PH. Especially at 12 hrs after PH, the immunogold labelling was significantly increased. Within the nucleus, gold particles appeared to be intensely distributed in areas of euchromatin and the nucleolus, while few gold particles were observed in the region of electron-dense heterochromatin (Figs. 8 & 9). 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, not rarely, the particles were localized near the rough endoplasmic reticulum. Time course of MT immunoreactivity revealed that distribution of gold particles in hepatocytes showed a highest level of MT immunoreactivity at 12 hr and then decreased at 48 hr (Fig. 10).

Liver regeneration, having presumably evolved to protect animals in the wild after the loss of hepatic tissue is a fundamental parameter of liver response to injury. This regeneration process is an orchestrated response induced by specific external stimuli and involving sequential changes in gene expression, growth factor production, and morphological structure (Matsumoto & Nakamura, 1998; Michalopoulos & 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 the 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 days post hepatectomy.

Despite many studies that have been carried out on MT during the past 40 years, 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 remnant liver after PH in rat.

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 intracellular processes. The nearly complete lack of mitochondrial labelling was common in all groups and these results are consistent with previous reports (Tohyama et al., 1993; Oh et al., 2004).

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 nucleus of 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 pro-

tein (Ghoshal & 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 & 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. These results suggest that MT possibly involves hepatocyte proliferation via the storage or the supply of various metal ions in the regenerating rat liver. Further studies are needed to evaluate the precise mechanism of MT gene induction and its physiological significance in regenerating liver.

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<국문초록>

진핵세포 내에 존재하는 metallothionein (MT)은 시스테인 함량이 높은 저분자량의 단백질로서 2가 금속이온들과의 친화력이 높아 중금속에 대한 해독작용, 금속이온의 대사 및 세포분열 등과 관련되어 있다. 본 연구자들은 간 재생능력이 우수한 흰쥐를 실험모델로 하여, 간의 70% 정도를 실험적으로 제거한 후, 간 재생을 유도하는 과정에서 시간 경과에 따라 간세포의 미세구조 변화와 더불어 MT 유전자 발현 및 이 단백질의 세포내 분포를 알아보고자 하였다. 부분 간 절제 후 간 조직이 증식되고 재구성되어 간이 원래의 크기로 재생되는 시간은 1주일 정도가 소요되었다. 재생중인 간세포는 핵대 세포질의 비가 크고, 핵내에 인이 크게 발달하고 크기가 작은 미토콘드리아가 다수 나타났으며, 조면소포체가 잘 발달하고 있었다. MT mRNA는 간 절제 직후부터 증가하기 시작하여 1시간 경과 후에 최대치에 이르렀고, 12시간 이후부터 감소하기 시작하였다. 재생중인 간세포에서 MT에 대한 면역반응은 간 절제 후 12시간이 경과한 군에서 가장 높게 나타났고, 이후 점차 감소하여 8일이 경과한 실험군에서는 정상 대조군과 유사한 정도로 감소하는 것으로 관찰되었다. 또한, 간 재생 초기에는 항 MT 금 입자들이 주로 세포질쪽에 분포하다가 점차 핵내에 존재하는 양이 증가하는 것으로 나타났다. 이러한 결과들은 간 절제 후 보상작용으로 일어나는 세포분열 단계에서 MT가 이 과정에 필요한 요소를 제공하는 역할을 수행하기 때문인 것으로 사료된다.

FIGURE LEGENDS

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

Figs. 2-5. Electron micrographs of regenerating rat liver tissues. Gly: glycogen, L: lipid droplet, Mi: mitochondrion, No: nucleolus, Nu: nucleus, RER: rough endoplasmic reticulum.

Fig. 2. The ultrastructure of hepatocyte in normal control. Mitochondria, smooth and rough endoplasmic reticulum and glycogen particles are seen to be typical.

Fig. 3. The ultrastructure of the hepatocyte at 4 hours after PH. There are well developed mitochondria around the nucleus. A few lipid droplets are seen and the glycogen particles become decreased.

Fig. 4. The ultrastructure of the hepatocyte at 12 hours after PH. It shows well developed rough endoplasmic reticulum and large lipid droplets, and also the sizes of mitochondria are decreased.

Fig. 5. The ultrastructure of the hepatocyte at 8 days after PH. A number of glycogen particles are distributed in cytoplasm and junctional complexes between adjacent cells are seen.

Figs. 6-10. Electron micrographs of immunogold labelling (arrow) of MT in the rat hepatocyte.

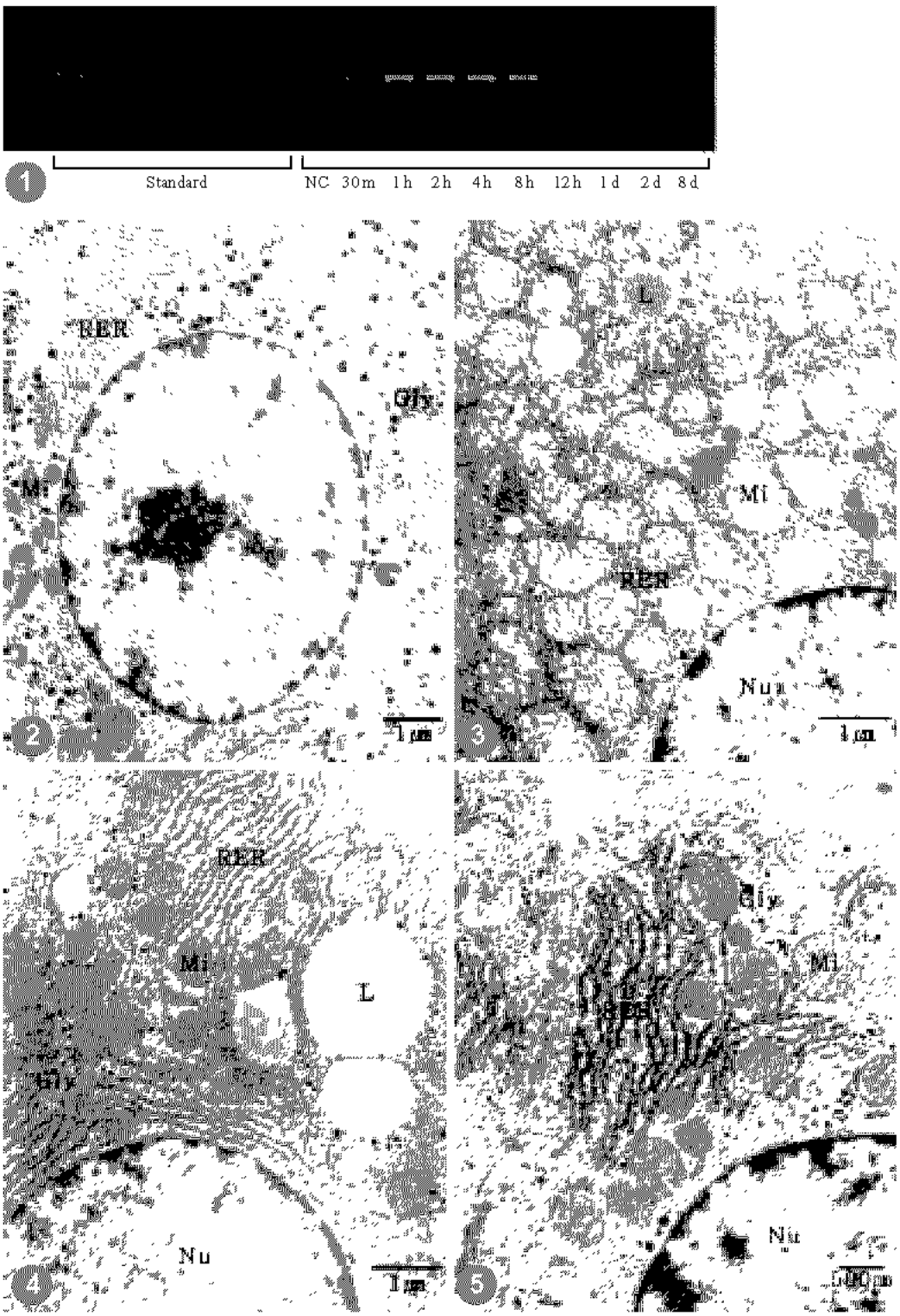
Fig. 6. Negative labellings for MT display in the nucleus in control (without primary anti-MT) at 24 hrs after PH. No gold particles are observed within the nucleus and the cytoplasm.

Fig. 7. In hepatocyte of sham control rat, A few gold particles are present adjacent to rough endoplasmic reticulum.

Fig. 8. This pattern of gold labelling is similarly seen in rat hepatocyte nucleus at 4 hr after PH. The gold particles are numerous at the nucleolus.

Fig. 9. Increased gold particles are ultrastructurally observed in hepatocyte nucleus, particularly adjacent to nucleolus of rat at 12 hr after PH. The gold particles indicating the labelling for MT are more distributed in the nucleus than in the cytoplasm.

Fig. 10. Immunogold labelling ind the hepatocyte at 48 hrs after PH. The distribution of gold particles are significantly decreased.


Standard
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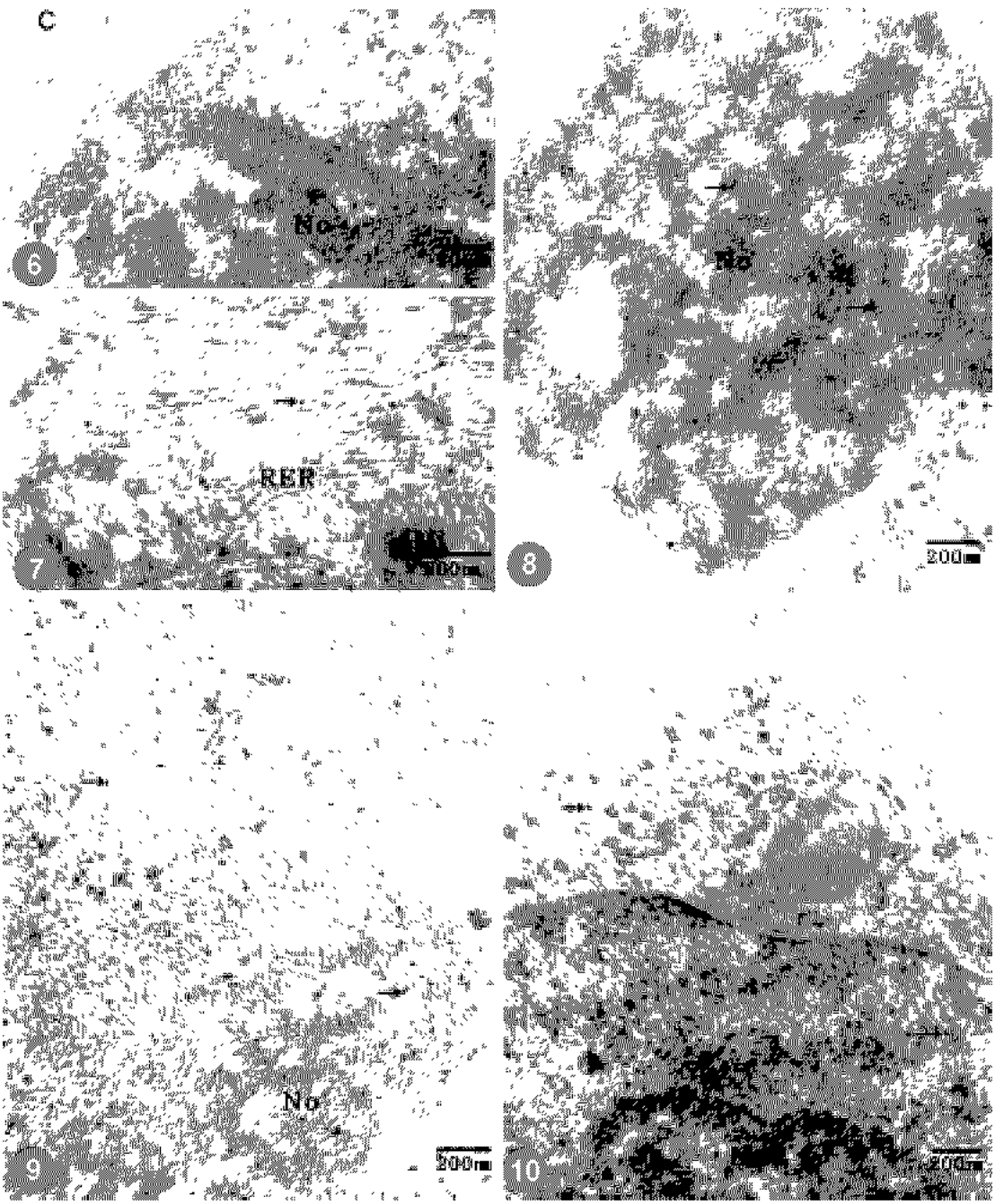
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