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Heterogeneous activation of protein kinase C during rat liver regeneration induced by carbon tetrachloride administration

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During rat liver regeneration induced by carbon tetrachloride administration, the protein kinase C α subspecies was activated in a heterogeneous fashion, a higher number of hepatocytes expressing the protein kinase C α subspecies being detected in the pericentral zone than in the periportal zone. This zonal heterogeneity became maximal at 24 h after the treatment. The distribution of hepatocytes expressing the protein kinase C α subspecies was roughly coincident with that of hepatocytes exhibiting DNA synthesis. These results suggest that protein kinase C may play a crucial role in liver regeneration.

Protein kinase C; Liver regeneration; Carbon tetrachloride administration; DNA synthesis

1. INTRODUCTION

Liver regeneration provides a good model for studying cell growth and differentiation in an 'in vivo' physiologically controlled situation. Various hepatotrophic factors exert their actions in regenerating liver by changing the intracellular concentrations of 'second messengers' such as Ca^{2+}, cyclic AMP and cyclic GMP [1-3]. These second messengers, in turn, have effects on specific enzymes, receptors and other proteins, leading to the generation of physiological responses. On the other hand, PKC plays a crucial role in signal transduction for a variety of biologically active substances which trigger cellular functions and proliferation [4]. This enzyme is present in various tissues and cell types [5], and is usually activated by 1,2-diacylglycerol, which is released during the process of phosphoinositide breakdown induced by receptor-mediated stimulation of many kinds of hormones, neurotransmitters and growth factors [6]. Accordingly, PKC may share the second messengers involved in liver regeneration and play an important role in mediating the biological actions of hepatotrophic factors. In this study, purification of PKC and immunohistochemical analysis were performed to determine whether, and if so how, PKC is activated in a hepatic lobule during liver regeneration induced by CCl_4 administration.

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Abbreviations: PKC, protein kinase C; CCl_4, carbon tetrachloride; BrdU, bromo-deoxyuridine; CLV, centrilobular vein; PV, portal vein.

2. MATERIALS AND METHODS

2.1. *Preparation of animals*

Fifty-one male Sprague-Dawley rats, weighing 200–250 g, were used in this study. In order to induce liver regeneration, forty-five rats were given a single intragastric dose, 0.5 ml, of a 1:1 (v/v) mixture of CCl_4 in mineral oil per 100 g body weight, intragastrically. No treatment except mineral oil administration (1 ml per 100 g body weight) was given to the remaining six rats, which formed the normal group. At 24 h, 48 h and 7 days after the treatment, three rats were processed for the partial purification of PKC. On the other hand, at 6 h, 12 h, 24 h, 48 h, 72 h and 7 days after the administration, three fasted rats were subjected to immunohistochemical analysis of PKC. In order to

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detect DNA replication after CCl_4 administration, the other three rats were injected intravenously with BrdU [7] at the dose of 30 mg/kg body weight 1 h before being killed. The rats in the normal group were similarly prepared. The protocol for this study was approved by the Animal Studies Committee of Osaka University.

2.2. *Enzyme purification and assays*

Rat liver PKC was purified and resolved into two fractions, types II and III, by chromatography on a hydroxyapatite column, which was connected to a high-performance liquid chromatography (Pharmacia FPLC) system, under the conditions described previously [8]. The type II and III PKCs correspond to the β I- plus β II- and α subspecies, respectively [9]. PKC was assayed by measuring the incorporation of $^{32}P_i$ into H_1 histone from [γ-^{32}P]ATP in the presence of phosphatidylserine (8 μg/ml), diolein (0.8 μg/ml) and $CaCl_2$ (0.5 mM) under the conditions given previously [8]. Basal activity was measured in the presence of 0.5 mM EGTA instead of phosphatidylserine, diolein and Ca^{2+}. Protein contents were determined by the method of Lowry et al. [10].

2.3. *Immunohistochemical analysis*

The procedures and conditions for immunohistochemical analysis in this study were described in detail elsewere [11,12]. Using synthetic oligopeptides specific for the predicted amino acid sequences of the C-terminal portions of the PKC α, β I- and β II-subspecies (α peptide residues 662–672; β I-peptide residues 661–671; β II-peptide residues 660–673), polyclonal antisera were raised in New Zealand White rabbits. Detailed procedures and properties of these antisera were described [13,14]. Sections from rats injected with BrdU were examined for DNA replication with a cell proliferation kit (Amersham, UK).

3. RESULTS

3.1. *Partial purification of PKC*

Fig.1 shows representative hydroxyapatite column chromatography of PKC from normal and treated rat livers. Two distinct types of PKC activity were obtained. The material of the first and second peaks from liver was eluted in fractions 30 through 38, and fractions 50 through 64, respectively, and this elution profile was highly reproducible. The activity of the material of the second peak increased to a peak at 24 h and then decreased, having returned to an almost basal level

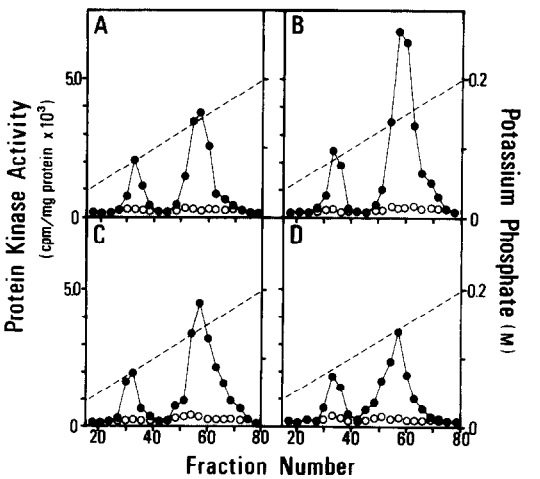


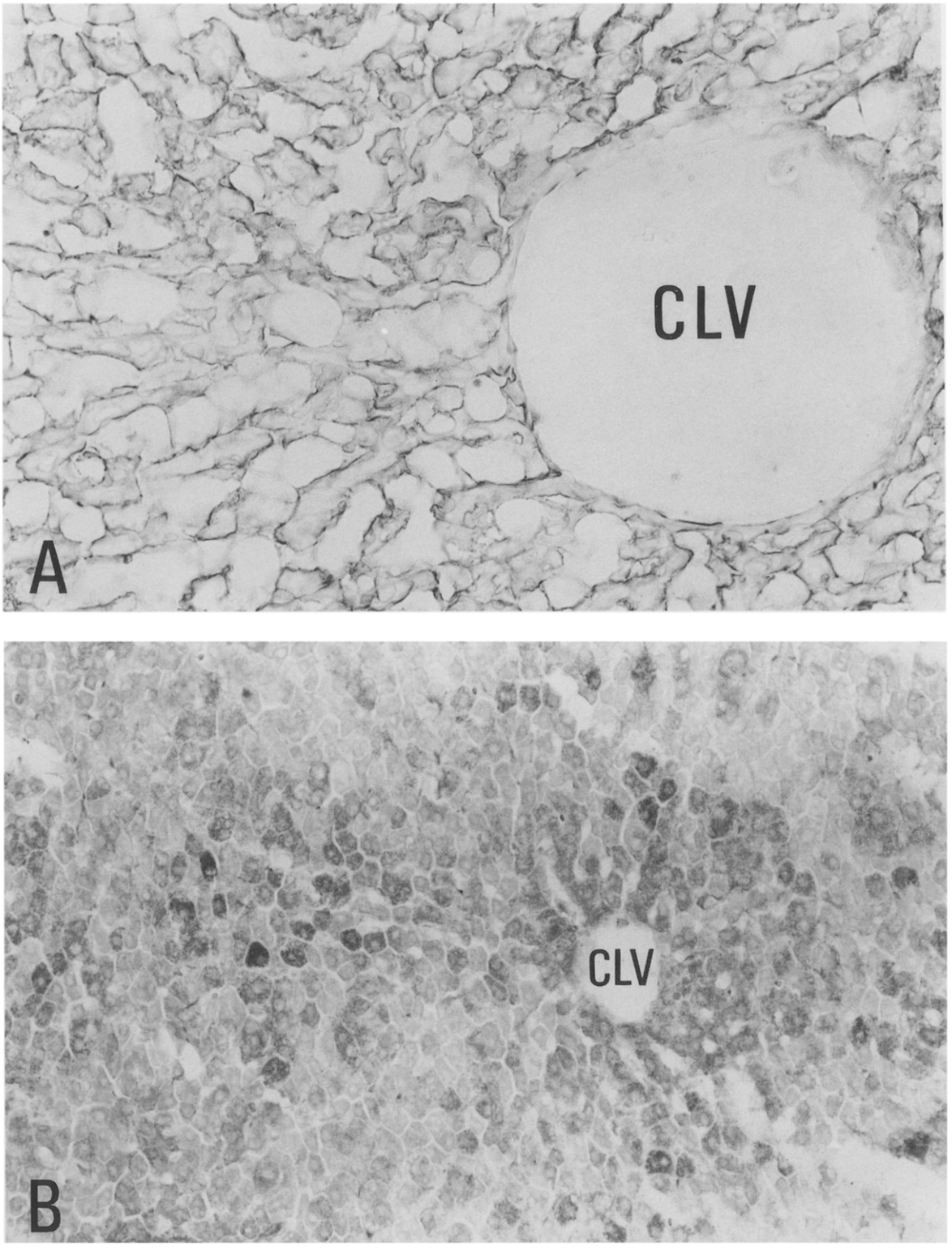
Fig.1. Hydroxyapatite column chromatography of PKC from normal and treated rat livers. Rat liver PKC was purified by DE-52 and hydroxyapatite column chromatographies, and the fractions from the hydroxyapatite column were assayed as described in section 2. PKC from normal liver (A), liver 24 h after CCl_4 administration (B), liver 48 h after CCl_4 administration (C), and liver 7 days after CCl_4 administration (D). (●—●) PKC activity in the presence of phosphatidylserine, diolein and $CaCl_2$; (○—○) PKC activity in the presence of EGTA instead of phosphatidylserine, diolein and $CaCl_2$; (– – –) potassium phosphate.

7 days after treatment, while that of the first peak material remained almost unchanged. The kinase activity in these fractions was almost entirely Ca^{2+}- and phospholipid-dependent.

3.2 *Immunohistochemical detection of PKC and DNA replication*

In normal liver, hepatocytes immunostained for the PKC α subspecies were diffusely distributed within a hepatic lobule (fig.2A). Immunoreactive structures were mainly localized in cell membranes of hepatocytes. The observation for the PKC β I- or PKC β II-subspecies was similar to that for the PKC α subspecies (data not shown). At 6 h after CCl_4 administration, heterogeneity of immuno-

Fig.2. Immunohistochemical localization of the PKC α subspecies in normal rat liver and rat liver at 6 h after CCl_4 administration. In normal liver, hepatocytes immunostained for the PKC α subspecies are diffusely distributed within a hepatic lobule (A). Immunoreactive structures are mainly localized in the cell membranes of hepatocytes. Heterogeneity of immunostaining first becomes evident at 6 h after the treatment. The few hepatocytes stained for the PKC α subspecies are detected predominantly in the pericentral and mediolobular zones (B). Immunoreactive structures in these cells are localized not only in the cell membrane but also throughout the cytoplasm. Magnification: × 90 in A; × 45 in B.


CLV
A
CLV
B

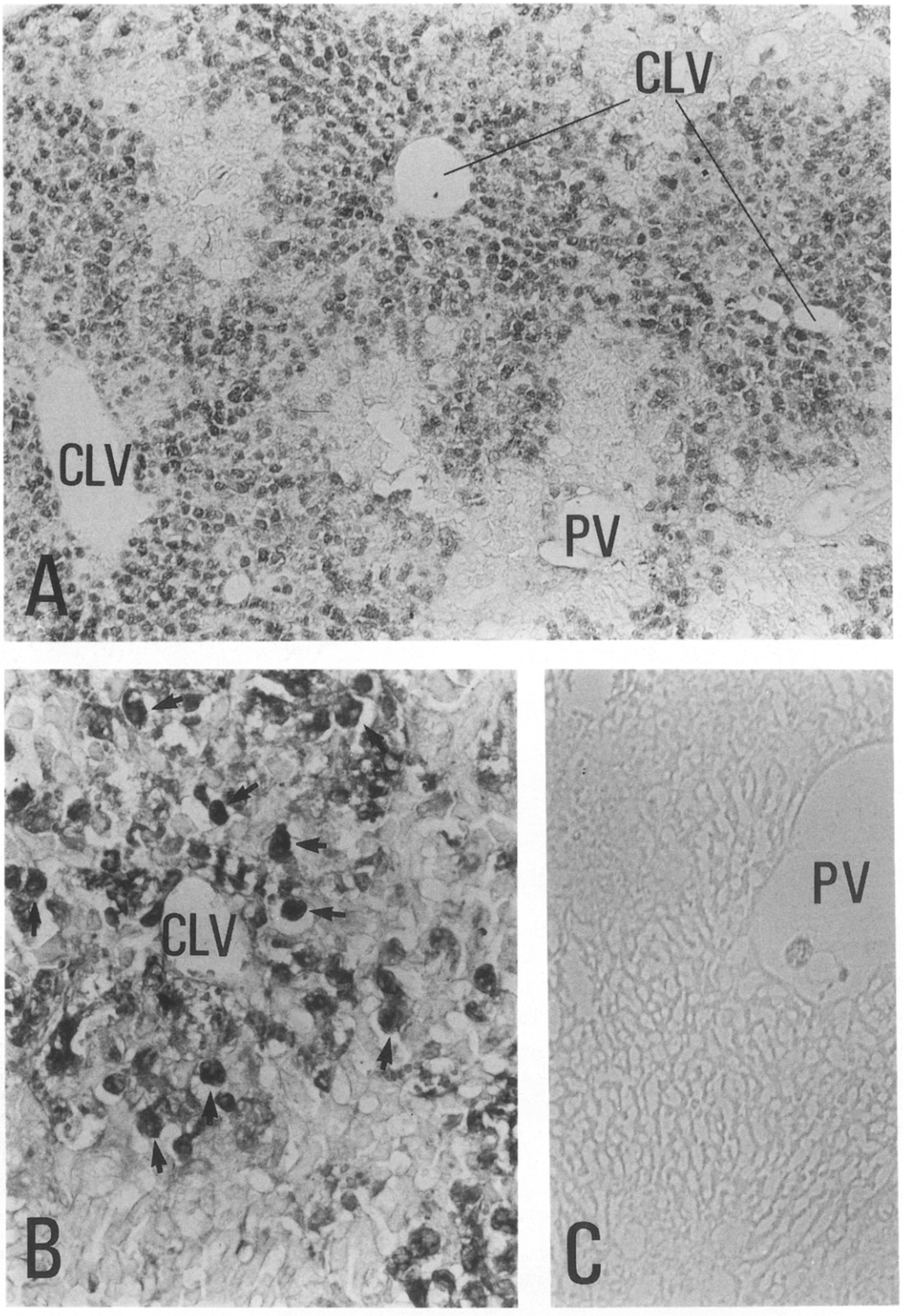

CLV
CLV
PV
A
CLV
B
PV
C

Fig.3. Immunohistochemical localization of the PKC α subspecies in rat liver at 24 h after CCl_4 administration. The number of hepatocytes immunostained for the PKC α subspecies has become maximal, with the development of clear zonal heterogeneity (A). Note that macrophages are also stained for the PKC α subspecies (B, arrows). Control reactions involving incubation with rabbit IgG instead of the first antiserum (C) and omission of the first antiserum (data not shown) are negative. Magnification: × 25 in A; × 50 in B, C.

staining first became evident. The few hepatocytes were intensely stained for the PKC α subspecies, predominantly in the pericentral and mediolobular zones (fig.2B). Immunoreactive structures in these cells were localized not only in the cell membrane but also throughout the cytoplasm. At 24 h after treatment, the number of hepatocytes stained for the PKC α subspecies (fig.3A) became maximal, with the development of clear zonal heterogeneity. Immunostained hepatocytes were observed around

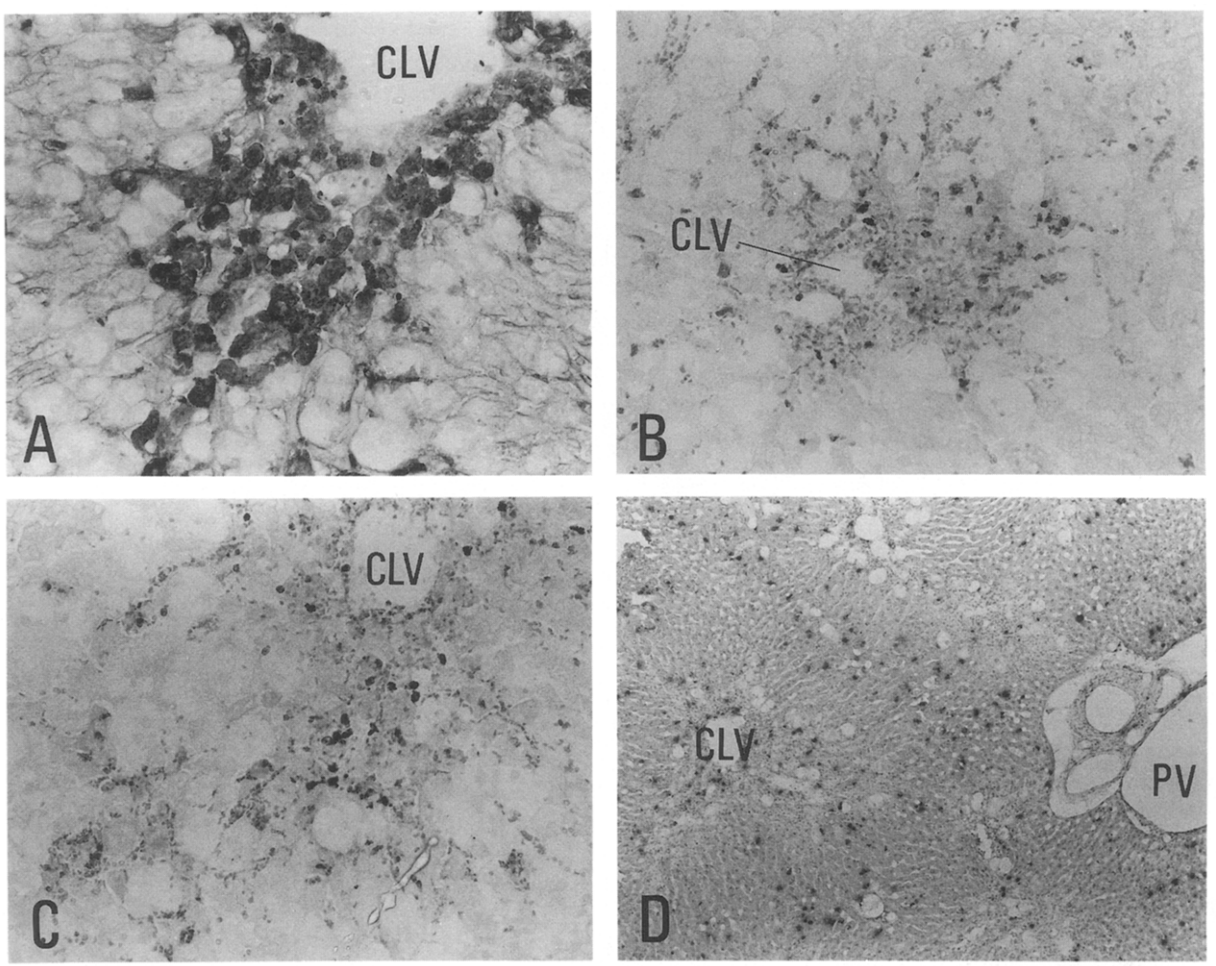


Fig.4. Immunohistochemical localization of PKC and BrdU in rat liver at 48 h after CCl_4 administration. Hepatocytes immunostained for the PKC α subspecies can be seen around areas showing a fatty change and/or ballooning in the pericentral and mediolobular zones (A). Heterogeneous staining is also evident for the PKC β I- (B) or β II- (C) subspecies, but the intensity of immunostaining is very weak compared with the case of the PKC α subspecies. The nuclei of some hepatocytes are immunostained for BrdU, which is indicative of ongoing DNA synthesis (D). The labeled nuclei are detected around areas showing pathological changes. Magnification: × 32.5 in A, B, C; × 16.3 in D.

areas where cell necrosis and/or a fatty change were observed. In addition, macrophages were also intensely stained for the PKC α subspecies (fig.3B, arrows). Control reactions involving incubation with rabbit IgC instead of the first antiserum (fig.3C) and omission of the first antiserum (data not shown) were negative. At 48 h after administration, hepatocytes immunostained for the PKC α subspecies were detected around areas showing a fatty change and/or ballooning in the pericentral and mediolobular zones (fig.4A). The number of immunostained hepatocytes was descreased, as compared with that at 24 h after treatment, while the zonal heterogeneity of immunostaining remained. A heterogeneous immunostaining pattern was also recorded for the PKC β I- (fig. 4B) or β II- (fig.4C) subspecies, whereas the intensity of immunostaining was weak compared with the case of the PKC α subspecies. On the other hand, the nuclei of some hepatocytes were stained for BrdU, which was indicative of ongoing DNA synthesis (fig.4D). The labeled nuclei were detectable predominantly around areas showing pathological changes in the mediolobular and pericentral zones. Finally, at 72 h after treatment, immunoreactive structures were only detected in the cell membrane of hepatocytes, as observed in normal liver (data not shown).

4. DISCUSSION

The present study is the first demonstration that the activity of the PKC α subspecies increased prior to the initiation of DNA synthesis during liver regeneration induced by CCl_4 administration. This change in the activity of the PKC α subspecies supported the finding on immunohistochemical analysis that the number of immunostained hepatocytes increased, with a peak at 24 h after CCl_4 administration, followed by DNA synthesis. Immunohistochemical analysis also showed that the PKC α subspecies was activated around areas showing pathological changes, and the distribution of hepatocytes expressing the PKC α subspecies was roughly coincident with that of those exhibiting DNA synthesis. Taking all the results together, it is suggested that the PKC α subspecies is the dominant PKC subspecies involved in crucial events regulating cell growth during liver regeneration. On the other hand, the present findings on PKC enzyme assaying and immunohistochemical analysis indicate that the contribution of the PKC β I- and β II-subspecies to liver regeneration is negligible, compared with that of the PKC α subspecies. The precise mechanisms underlying activation of the PKC α subspecies and its functional roles in hepatocytes during liver regeneration remain unknown. Some of the hepatotrophic factors would cause the receptor-mediated hydrolysis of inositol phospholipids, resulting in activation of PKC. Vasopressin, angiotensin and epinephrine, defined as competence factors for cellular growth [15], may activate PKC, and then the activated PKC may mediate the sequence of early events in cellular proliferation of the liver, as speculated for various tissues and cell lines [6,16–21]. In addition, the activated PKC modulates binding and the phosphorylation of receptors for EGF [22,23], which is defined as a progression factor [15]. The modulation of the EGF receptor by PKC may provide a mechanism whereby competence factor(s) and progression factor(s) can act sequentially in stimulating cell growth. In this way, activation of PKC during liver regeneration may mediate and regulate the sequence of events concerning the competence and progression of cellular proliferation of the liver. Further investigations are required to clarify the functional role of the PKC α subspecies during liver regeneration.

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