***RB* gene product levels measured by a specific radioimmunoassay decrease in regenerating rat liver after partial hepatectomy**

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ABSTRACT

We have analyzed quantitatively the retinoblastoma susceptibility gene (*RB*) product (*RB*-P) in regenerating rat liver by a specific radioimmunoassay. The assay system was developed using a synthetic peptide corresponding to the 901-928 sequence of *RB*-P as haptenic immunogen and standard antigen, ¹²⁵I-4-hydroxyphenylpropionyl *RB*-P (901-928) as tracer and anti-*RB*-P (901-928) serum R0811. The antiserum proved to recognize a 110 kDa component in rat liver extract by immunoblot analysis. *RB*-P in regenerating rat liver measured by the radioimmunoassay (*RB*-P-IR) was found to decrease significantly on day 1 (to $53.5 \pm 11.6\%$, mean $\pm$ SEM, $P < 0.01$), day 2 ($74.8 \pm 11.3\%$, $P < 0.05$), day 3 ($75.1 \pm 11.2\%$, $P < 0.05$) and day 4 ($61.0 \pm 10.2\%$, $P < 0.01$). The decrease was most pronounced on day 1 when DNA synthesis of hepatocytes is most active during regeneration. The level of *RB*-P-IR returned to the control level on day 6 when liver regeneration was almost completed. The time course of the reduction in *RB*-P-IR roughly paralleled that of the increase in *c-myc* product measured by a radioimmunoassay in our previous study (14). The present study revealed a quantitative change in *RB*-P-IR during the course of rat liver regeneration after partial hepatectomy which involves a highly synchronized replication response of a major population of hepatocytes.

The retinoblastoma susceptibility gene (*RB*) has been known as a growth suppressor gene. The product of the gene (*RB*-P) has been detected in all mammalian cells examined (3), and the protein has been implicated to play a role in normal cell growth (1, 3, 16). Biochemical and immunohistochemical studies have demonstrated the nuclear localization of *RB*-P (16, 19, 25) and the protein, like *c-myc*, *N-myc*, *c-myb* and *c-fos* proteins, is accepted as a nuclear protein with DNA binding activity (26). Regeneration of rat liver after partial resection has been used for studies of growth- and proliferation-related gene expression in normal cells under *in vivo* condition (4, 7, 9, 10, 12, 13, 17, 22-24). We have previously shown the characteristic increases in immunologically measurable *c-myc* and *c-fos* products (*c-myc*-P-IR and *c-fos*-P-IR, respectively) in regenerating rat liver after partial resection (14).

In the present study, we developed a radioimmunoassay specific to *RB*-P using synthetic peptides, and examined changes in *RB*-P in regenerating rat liver after partial hepatectomy. The synthetic peptide used as haptenic immunogen corresponds to the C-terminal 901-928 sequence of *RB*-P which has been predicted to be one of the most hydrophilic regions of the molecule (8, 15, 16).

Adult male Wistar rats (180-200 g) were subjected to partial (70%) hepatectomy or sham-operation under ether anesthesia by the method of Higgins and Anderson (11). Liver tissue was extracted by homogenizing with a Bio-Mixer model BM-1 in 5 vol of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 5% glycerol, 50 mM NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF), 250 KIU/ml aprotinin, 0.01% soybean trypsin inhibitor (SBTI), 0.1% bacitracin and 50 mM EDTA. The homog-

enate was centrifuged at 160,000 *g* for 1 h at 4°C and the supernatant was taken. Protein concentration was determined by the method of Bradford (2). Gel filtration of the tissue extract was carried out on a column (1.6 × 96 cm) of Sepharose 6B with 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 50 mM EDTA, 1 mM PMSF, 0.02% NaN₃ and 0.01% SBTI. Fractions of 3.7 ml each were collected. Syntheses of *RB*-P (901-928) and *N*^α-4-hydroxyphenylpropionyl *RB*-P (901-928) were carried out by solid phase method using an automated peptide synthesizer ABI model 430A. *RB*-P (901-928) of high purity (4.4 mg) was conjugated with porcine thyroglobulin (27.3 mg) in the presence of water-soluble carbodiimide (25.4 mg). The conjugate (5.0 mg) emulsified with complete Freund's adjuvant (1.5 ml) was injected to three rabbits intradermally. Injection with the conjugate was repeated every 2 weeks using a half of the initial amount of immunogen. Antiserum R0811 thus obtained was used for immunoblot analysis and radioimmunoassay. 4-Hydroxyphenylpropionyl *RB*-P (901-928) was radioiodinated by the chloramine T method. Radioimmunoassay was performed according to the method previously described (14), using antiserum R0811 (final dilution, × 10,500), ¹²⁵I-*N*^α-4-hydroxyphenylpropionyl *RB*-P (901-928) as tracer and *RB*-P (901-928) as standard antigen. The standard diluent used for the assay was 10 mM phosphate buffer (pH 7.4) containing 0.5% bovine serum albumin, 50 mM EDTA, 0.01% SBTI, 0.1% bacitracin, 0.1 mM PMSF and 0.14 M NaCl. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of rat liver extract were carried out as previously described (14). Antiserum R0811 (dilution, × 3,000) was used for immunoblot analysis.

Fig. 1 shows a typical standard curve in the present radioimmunoassay for *RB*-P together with a serial dilution curve of rat liver extract which was linear and parallel to the standard curve. The assay system was able to quantitate *RB*-P as low as 80 fmol. The component measurable by the radioimmunoassay system was eluted as a peak in gel filtration as shown in Fig. 2. Immunoblot analysis of this peak fraction as well as crude liver extract showed a single band corresponding to molecular weight of 110 kDa (Fig. 3). The results indicate the existence of immunochemically detectable *RB*-P (*RB*-P-IR) in rat liver and prove the validity of the present radioimmunoassay for measurement of *RB*-P in crude rat liver extract. The change in *RB*-P-IR level during liver regeneration after partial resection is shown in Fig. 4. *RB*-P-IR level decreased sig-

Fig. 1 A typical standard curve (—●—) of a radioimmunoassay for *RB*-P with a dilution curve of rat liver extract (—▲—)

Fig. 2 Gel filtration profile on a Sepharose 6B column of *RB*-P-IR in rat liver extract

nificantly on day 1 (to $53.5 \pm 11.6\%$, mean $\pm$ SEM, $P < 0.01$), day 2 ($74.8 \pm 11.3\%$, $P < 0.05$), day 3 ($75.1 \pm 11.2\%$, $P < 0.05$) and day 4 ($61.0 \pm 10.2\%$,

Fig. 3 Immunoblot analysis after SDS-polyacrylamide gel electrophoresis of rat liver extract (1) and the peak fraction of gel filtration (Fig. 2) of rat liver extract (2)

$P < 0.01$). The changes in *RB*-P-IR at 3, 6, 12 and 18 h after resection were statistically not significant.

RB-P-IR was found to decrease during rat liver regeneration after partial resection. The decrease was most pronounced at 24 h after resection when DNA synthesis in hepatocytes is known to be most active. The lowered level of *RB*-P-IR in regenerating liver persisted until day 5. Proliferation of non-parenchymal cells lagged behind hepatocytes in time during regeneration (5, 6). The persistent decrease in *RB*-P-IR level with another trough on day 4 may relate to the proliferation of these cells. The level of *RB*-P-IR returned to normal on day 6 when liver regeneration was almost completed. The present study revealed an inverse relationship between the concentration changes in *RB*-P-IR and in *c-myc*-P-IR and *c-fos*-P-IR in regenerating rat liver. The time course of the reduction in *RB*-P-IR roughly paralleled that of *c-myc*-P-IR increase (14). Mihara and coworkers (19) have observed by an immunoprecipitation method using anti-synthetic peptide antisera that the amount of *RB*-P per cell was increased as the cells progressively matured through the G_1 , S and $G_2 + M$ phases of the cell cycle in HL-60 cells. However, the experimental materials and conditions used by Mihara and colleagues differed from those of the present study. Rustgi and colleagues (21) have suggested that *c-myc* and *RB* products cooperate through direct binding to control cell proliferation. Negative regulation of human *c-fos* expression by *RB* product has also been observed in NIH/3T3 cells cotransfected

Fig. 4 Time course of percent change in *RB*-P-IR level in regenerating rat liver after partial hepatectomy (mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$)

with *RB* protein expression plasmids with *c-fos* promoter constructs (20).

Studies on the regulation mechanism of *RB*-P activity have focused almost exclusively on the phosphorylation state of the protein. It has been reported that *RB*-P switches between hyperphosphorylated and hypophosphorylated states in a cell cycle-specific manner (18, 26). The present study revealed a quantitative change in *RB*-P immunohistochemically measured during the course of rat liver regeneration after partial hepatectomy which involves a highly synchronized replication response of a major population of hepatocytes. The decrease in *RB*-P-IR concentration in regenerating liver observed during the stage when hepatocytes and nonparenchymal cells are growing and proliferating seems to suggest the existence of a mechanism of the regulation of *RB*-P activity implicating quantitative changes in the protein.

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