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REGENERATING LIVERS OF OLD RATS CONTAIN HIGH LEVELS OF C/EBPα AND HAVE ALTERED EXPRESSION OF CELL CYCLE ASSOCIATED PROTEINS.

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Running Title: Regulation of C/EBPα and p21 is deranged in old animals.

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SUMMARY

The proliferative response to partial hepatectomy (PH) has been shown to be reduced and delayed in old animals. The nuclear transcription factor, CCAAT/Enhancer Binding Proteins α (C/EBPα) is expressed at high levels in liver and inhibits growth in cultured cells. Here we present evidence that the expression of C/EBPα in old (24 mo) rats differs from its expression in young animals (6-10 mo) during liver regeneration. Induction of Proliferating Cell Nuclear Antigen (PCNA), a marker of DNA synthesis, occurs at 24 hrs after (PH) in young rats. In old animals, the induction of PCNA is delayed and reduced, consistent with previously published observations showing a delay of DNA synthesis in old rats. In young rats, the normal regenerative response involves a reduction of 3-4 fold in the levels of C/EBPα protein at 3-24 hours. In old animals, C/EBPα is not reduced after PH. Induction of C/EBPβ, another member of the C/EBP family, is delayed in old animals. Changes in the expression of C/EBP proteins are accompanied by alteration of several cell cycle associated proteins. The CDK inhibitor, p21, is also decreased in young rats after PH and correlates with C/EBPα reduction, but in old animals, no reduction of p21 is observed. p21 reduction is specific as the amounts of other CDK inhibitors, p16 and p27, were not altered during liver regeneration. Induction of the mitotic specific protein, cdc2 p34, is 3-4 fold less in regenerating liver of old rats than in the liver of young animals, confirming the reduced proliferative response in old animals. We suggest that the failure to reduce the amount of C/EBPα and p21 is a critical event in the dysregulation of hepatocyte proliferation in old animals following PH.

INTRODUCTION

The effect of age on liver regeneration in rats and mice has been the subject of numerous investigations showing that the proliferative response to partial hepatectomy in older rats is reduced and delayed (1,2). The rate of DNA synthesis in young rats is biphasic with peaks at 22-24 hrs and 35-37 hrs after PH. In old animals, a delayed, single and broad peak of DNA replication initiating at 30-36 hrs was observed. The levels of DNA polymerase α and DNA replication in regenerating livers of aging mice were also reduced and delayed compared to young animals (3). Recently we demonstrated that C/EBPα is a crucial regulator of liver proliferation: hepatocytes in C/EBPα knockout mice had increased DNA synthesis at birth compared to normal animals and hepatocyte proliferation continued at a high rate (30-40%) relative to control littermates (3%) at day 17 of life (4). The observations of this genetic knockout model and the reported delay in regenerative response in aged animals prompted our examination of the regulation of C/EBPα expression in livers of old rats. The nuclear transcription factor, C/EBPα , a member of the bZIP family of proteins, is expressed at high levels in differentiated hepatocytes and plays a significant role in adipose tissue differentiation (5-8) and in control of cell proliferation. Overexpression of C/EBPα in cultured mammalian cells leads to growth arrest (5,9-11). Although C/EBPα mediated growth arrest of mammalian cells is well documented, the molecular mechanisms by which this factor inhibits growth are not well understood. One pathway of C/EBPα mediated growth arrest was suggested by experiments with a stably transformed human cell line that conditionally expressed high levels of C/EBPα (10). C/EBPα inhibited growth of these human fibrosarcoma cells through the stabilization and elevation of p21 protein level (10). Recently we have shown that the hepatocytes of newborn C/EBPα -/- mice have several characteristics specific to dividing hepatocytes: increased DNA

synthesis, low levels of p21 and induced levels of S-phase specific cdk2/E2F complexes (4). The p21 protein, an inhibitor of DNA synthesis and cyclin dependent kinases (CDKs), was discovered as an mRNA that was overexpressed in senescent fibroblasts (12). p21 is also known as SDI-1 (senescent derived inhibitor-1) (12), WAF-1 (wild type p53 activated fragment 1) (13) and as CIP-1 (CDK interacting protein 1) (14). Investigation of p21 function carried out in cultured cell lines. has shown that the general mechanism of p21 action is inhibition of cyclin dependent kinases resulting in growth arrest in the G1 phase of cell the cycle (15). p21 has been also shown to interact with Proliferating Cell Nuclear Antigen (PCNA), a protein that is involved in DNA synthesis, and to inhibit proliferation through this interaction (16). p21 mediated inhibition of CDK activities is complex and depends on whether the p21/cdk complexes contain one p21 molecule (this complex is active) or two molecules of p21 (kinase activity is not present) (17,18).

Here we present evidence that the regulation of C/EBPα and p21 expression in hepatocytes in old rats (24 mo) following partial hepatectomy shows no reduction in the levels of these proteins unlike their young (6-10 mo) counterparts. Failure to reduce the p21 and C/EBPα in old rats is coincident with alteration in the expression of a number of cell cycle associated proteins. We propose that the failure to lower C/EBPα and p21 protein levels is responsible for the delay in the synthesis of DNA that is observed in old rats relative to young.

EXPERIMENTAL PROCEDURES

Animals and partial hepatectomy. Fischer 344 rats of 6-10 mo (young) and 24 mo (old) age were used in these studies. Approximately 70% of the liver was surgically removed and regeneration was allowed to proceed for 30 min, 3, 6, 12, 24 and 48 hrs. Three animals at each time point were used for young and old rats. Only 2 old rats were investigated at 24 hours and a single old animal at 48 hrs. Animals were sacrificed under anaesthesia. Livers from untreated animals were used as the point 0 controls. Sham operations were performed in parallel to the hepatectomized animals and served as a control for the stress response.

Protein Isolation and Western analysis. Isolation and analysis of proteins from HT1080 and HT1 cultured cells were described in our previous publication (10). HT1 cells were treated with IPTG (inducer of C/EBPα expression) or with Glucose (control) for 1, 2 and 3 days. Whole Cell Extracts (WCE) were isolated and analyzed by Western blotting. Liver nuclear extracts (NE) were isolated as follows. Livers were homogenized in buffer A containing 25 mM Tris-HCL, pH 7.5, 50 mM KCL, 2mM MgCL2, 1mM EDTA and 5mM DTT. Nuclei were pelleted by centrifugation at 5000 rpm for 10 min and washed with buffer A. Supernatant (cytoplasm) was frozen. High salt extraction of proteins was performed by incubation of nuclei with buffer B [25 mM Tris-HCL, pH 7.5, 0.42 M NaCL, 1.5 mM MgCL2, 1mM DTT, 0.5 mM EDTA and 25% sucrose] for 30 min on ice. After centrifugation, the supernatant (nuclear extract) was divided onto small fractions and kept at -80°C. Western analysis was carried out as described (10). Briefly, 50-100 ug of nuclear proteins were loaded on 12% polyacrylamide -0.1%SDS gel. A 15% gel was used for low molecular weight proteins. After separation, proteins were transferred onto membranes (NitroBind, Micron Separation, Inc.) using electro blotting. To equalize the protein loading, a preliminary filter was

stained with coomassie blue to verify the measured protein concentration. After detection of specific proteins, each filter was reprobed with antibodies to β-actin (Sigma) or with antibodies to cdk4. The β-actin control, shown for Westerns presented in the paper, was used for quantitation of protein expression in young and old rats. The level of the proteins was calculated as the ratio to β-actin. Filters were blocked by 10% dry milk, 2% BSA prepared on TTBS (20mM Tris-HCL, pH 7.5, 150 mM NaCL and 0.05% Tween-20) buffer saline. Incubations with primary and secondary antibodies were carried out according to recommendations for each antibody. 0.5% dry milk was added to TTBS and this solution was used for incubation with antibodies. For analysis of the p21 protein, two different antibodies were used: a monoclonal antiserum, cp36 (gift from W. Harper, S. Elledge and E. Harlow) and a polyclonal antiserum M-143 (Santa Cruz Biotechnology). All antibodies showed similar results. Antibodies to C/EBPα (14AA), C/EBPβ (C-19), PCNA, cdk4, cdk2, p53, p27, cdc2 p34 were from Santa Cruz Biotechnology. Antibody to p16 was a gift of Dr. C.J. Sherr. Immunoreactive proteins were detected using the enhanced chemiluminescent (ECL) protocol (Amersham).

Electrophoretical mobility shift assay. Conditions for binding reactions were described in our previous publications (10,19). Antibodies and unlabeled competitors were added to binding reactions before probe addition. The sequence of the USF oligonucleotide was described (19). Oligonucleotide probe for Sp1 was from Santa Cruz Biotechnology.

RNA Isolation and Northern analysis. Total RNA was isolated as described (10). Twenty five ug of total RNA were loaded on 1%-agarose:2.2M formaldehyde gel, transferred onto a membrane and hybridized with specific probes. Each filter was hybridized sequentially with C/EBPα, C/EBPβ and 18S rRNA specific probes as described (10). Quantitation of Northerns was performed using

phosphor imaging. The levels of C/EBPα and C/EBPβ mRNAs were normalized to the 18S rRNA control.

Immunostaining with antibodies to PCNA. Tissue was fixed overnight in 10% formalin, paraffin embedded and stained for PCNA using anti-PCNA (Dako) and AEC (Vectastain).

RESULTS

The proliferative response after partial hepatectomy is delayed in old rats.

A reduction in C/EBPα expression during liver regeneration in rats has been previously described (20-22). Because the reduction in C/EBPα precedes DNA replication in young animals, it has been speculated that the levels of C/EBPα in the normal liver must be reduced for cell proliferation to occur. To investigate the effect of aging on C/EBPα in liver, we measured this protein by Western blotting in young (6-10 months, 3 per time point) and old rats (24 months). Sham operated rats served as the control. We initially verified that the proliferative response is reduced and delayed in old animals after PH. In young rats, DNA synthesis is reported to take place between 16 and 26 hrs with a peak at 22 to 24 hrs after PH (1,23). The proliferative response in the young and old animals in our study was investigated by measuring PCNA expression using immunostaining of tissue sections (Fig. 1) as well as Western blotting (Fig. 2). The number of PCNA-positive hepatocyte nuclei at 24 hrs after PH clearly increases in young, but not in old animals (Fig. 1). In old rats, PCNA positive nuclei are detectable at 48 hrs indicating a delayed response to PH. Western analysis of PCNA (Fig. 2, upper part) shows that PCNA levels were increased at 24 hrs after surgery in young animals, indicating a normal proliferative response. However, PCNA expression in old rats is decreased at 3 and 6 hrs and returns to control levels at 24 hrs. A slight induction is observed only at 48 hrs. Thus, both the number of cells entering S-phase and the total amount of PCNA are reduced in old rats relative to the young animals after partial hepatectomy.

The levels of C/EBPα protein are not reduced in the livers of old rats in response to the proliferative stimulus of PH.

Reduction of C/EBPα in young animals in response to PH has been described by several groups (20-22). To determine whether the levels of C/EBPα protein were similarly reduced in old animals, we examined C/EBPα protein levels at different times after PH in these animals. Three animals were studied for most time points. Fig. 2 shows a representative Western analysis. C/EBPα mRNA produces two C/EBPα isoforms, 42 kD and 30 kD, presumably by a "leaky ribosome scanning" mechanism (24-26). A reduction of both C/EBPα isoforms in young rats was observed by 3 hrs after PH with maximum decrease at 12-24 hrs, in good agreement with previously published observations (20-22). The pattern of C/EBPα expression in old animals is quite different. C/EBPα levels are not reduced. In some old animals, C/EBPα induction was observed at 3 and 6 hrs (data not shown). Sham operated animals did not show changes in C/EBPα expression. There was little variation in C/EBPα expression among the animals at each time point although a slight reduction of C/EBPα was detectable in one old rat at 24 hrs after PH. Thus, the hepatocytes of old rats were shown to maintain C/EBPα levels, unlike the situation in younger animals.

p21 is reduced in hepatocytes of young rats during liver regeneration, but fails to decline in old rats.

C/EBPα is an inhibitor of proliferation in cultured cells and in developing liver and regulates the level of p21 protein (4,10). We speculated that the expression of p21 protein might be changed in old rats because of the alterations in the response of C/EBPα to PH. Fig. 3 shows a representative Western with two sets of young and old rats. Sham operated animals were used as the control and

these did not show changes in p21 expression after surgery. The p21 level in young rats was dramatically reduced at 3-24 hrs, however, in old animals no reduction was observed. Some old animals showed a slight induction of p21 at different times after PH (Fig. 3). We have also compared expression of p27 and p16 in young and old rats and did not detect changes in their expression (data not shown). Thus, in hepatocytes of old rats, p21 protein does not decline in response to PH. This observation is consistent with a C/EBPα mediated regulation of p21 in liver and suggests that the altered regulation of C/EBPα and p21 in old rats after PH in tern changes the kinetics cell cycle progression.

Protein levels and phosphorylation of cdc2 p34 are altered in old rats.

Having observed steady levels of expression of C/EBPα and p21, and alteration of PCNA expression in old rats, we examined the level of the cdc2 p34 protein as a second measure of the proliferative status of the cells. This kinase plays an important role in the transition of cells through G2 to M phase (27,28). Cdc2 p34 is not detectable in quiescent hepatocytes from either young or old rats. Within 48 hrs following PH, a strong induction of cdc2 p34 is observed in young rats, however, in old rats the induction is moderate (Fig. 4A). The induction of cdc2 p34 in old rats is 3-4 fold less than in young rats. In addition, inducible cdc2 p34 protein in young rats consists of two bands, whereas cdc2 p34 in old rats migrates as a single protein. The upper immunoreactive band seen in regenerating livers is the phosphorylated form of cdc2 p34 as treatment of proteins with increasing amount of alkaline phosphatase (AP) resulted in a shift of the upper band to the position of the bottom one (Fig. 4A). This observation shows that expression and phosphorylation of cdc2 p34 differs in young and old rats and reflects the degree of proliferation induced by PH.

To test a causal effect of C/EBPα on the expression of cdc2 p34 in vitro, we measured the level of cdc2 p34 protein in human HT1 cells. We have previously shown that forced expression of C/EBPα inhibits proliferation of HT1 cells via elevation of p21 protein (10). If cdc2 p34 is under C/EBPα:p21 regulation, the level of cdc2 p34 should be decreased in HT1 cells after induction of C/EBPα and p21. Western analysis of proteins from cells without C/EBPα (G) and with (I) is shown in figure 4B. The upper panel shows the expression of C/EBPα in response to IPTG. The cdc2 p34 protein levels are reduced in the HT1 cells expressing high levels C/EBPα at days 2 and 3. Expression of cdc2 p34 is not changed in response to IPTG addition in the control HT1080 cells that do not contain an inducible C/EBPα gene and do not express endogenous C/EBPα. The same filter was stripped and reprobed with antiserum to cdk4. No change in cdk4 was observed, indicating a specific reduction of cdc2 p34. Thus, high levels of C/EBPα bring about growth arrest and lead to depression of cdc2 p34 expression. These observations are consistent with the thought that the low level of cdc2 p34 in old rats reflects the decreased proliferative response to PH..

C/EBPβ expression in young and old rats inversely correlates with C/EBPα.

In order to understand the basis for abnormal regulation of C/EBPα in old rats, we searched for nuclear factors that can directly regulate C/EBPα expression in liver. We studied the expression of DNA binding proteins that interact with the rat C/EBPα promoter. The rat C/EBPα promoter has been shown to contain high affinity binding sites for nuclear factors: USF, C/EBP proteins and Sp1 and these binding sites are occupied by proteins isolated from rat liver (29,30). Electrophoretic mobility shift with specific antisera showed no detectable differences in young and old animals in USF and Sp1 expression (data not shown). However, C/EBPβ expression differs significantly.

Several published studies showed that, in young rats, expression of both C/EBPβ translation isoforms, LAP and LIP, is induced at 3 and 6 hrs after PH (21,22). Northern analysis with total RNA from young and old rats isolated at various times after PH showed that, in young rats, induction of C/EBPβ mRNA was consistent with previously published observations. In old rats, however, C/EBPβ mRNA was not induced within this 3 to 6 hr time frame, but was shifted to 12 -24 hrs after surgery (Fig. 5A). Reprobing the filter for C/EBPα shows an inverse pattern of mRNA expression. When C/EBPβ mRNA induction is at its maximum, C/EBPα is reduced. The expression of C/EBPβ protein isoforms in young and old rats is in agreement with the expression of mRNA. Both C/EBPβ isoforms (LAP and LIP) are induced in old rats at the later times (12-24 hrs) (Fig. 5B), but to higher levels. Thus, although induction of C/EBPβ protein is delayed in old animals compared to young, the amount of induction is greater. It is interesting to note that the truncated C/EBPβ isoform, LIP, is induced at higher levels than LAP resulting in a change of the LAP/LIP ratio (Fig. 5B). The relative abundance of C/EBPβ isoforms in young and old rats was approximately 1:1 suggesting that LIP levels are adequate to inhibit the activity of other bZIP proteins by hetero dimerization and may thereby inhibit genes with promoters containing C/EBP consensus sites (see Discussion).

DISCUSSION

Liver regeneration is affected by the age of the animal: in old animals, the proliferative response to PH was significantly delayed although full restoration of liver mass eventually occurred (1,2). While the molecular basis for this delay is not known, alterations in the expression of numerous proteins have been well documented in regenerating hepatocytes of young animals (23). C/EBP proteins are abundant in liver and are good candidates for regulation of hepatocyte proliferation because of their role in growth arrest of cultured cells (5,9-11). Their growth inhibitory function in vivo, however, is not clearly understood. C/EBPα is highly expressed in quiescent hepatocytes and is reduced when hepatocytes undergo division (20-22). Because C/EBPα is an inhibitor of cell proliferation, the high levels of C/EBPα in regenerating livers of old rats might well affect the expression of cell cycle associated proteins. C/EBPα has been shown to elevate the protein levels of p21 in cultured cells (10). Our results with newborn and suckling C/EBPα knockout mice showed that, in the absence of C/EBPα, p21 levels are greatly reduced and hepatocyte proliferation is increased at birth and continues for up to 2 weeks, at which time, genetically normal hepatocytes have a low level of proliferation (4). Here we describe the expression of p21 protein during regeneration and show that p21 (but not p27 or p16) is likely to be an important regulator of hepatocyte proliferation. Another in vivo association of hepatocyte proliferation and p21 levels has been described by Wu et al.; transgenic animals overexpressing p21 have reduced proliferative response to PH (31). Based on these observations we propose that C/EBPα regulates hepatocyte proliferation during the neonatal period of development. In this paper, we show that regulation of C/EBPα expression in old rats is deranged following the regeneration stimulus of PH. Consistent with previously published data, the proliferative response in the old animals under study is reduced and delayed (Fig. 1). The expression

of the cell cycle associated proteins, (PCNA and cdc2 p34), putative targets of the C/EBPα:p21 pathway, is altered. In addition to the observation that older animals failed to reduce C/EBPα protein levels in response to PH, the C/EBPα mRNA was shown to be 2-3 fold induced in old animals at 3 hrs after PH. Because expression of C/EBPα mRNA during liver regeneration has been shown to be regulated at the level of transcription (22), we suggest that old animals have lost the appropriate transcriptional control of this gene. Our analysis of transcription factors USF and Sp1 that have been reported to interact with the C/EBPα promoter did not detect significant differences between the age groups in the expression or in the binding activities of these proteins. However, many factors are likely to regulate C/EBPα expression in the liver and future studies will address this issue.

The proximal region of rat C/EBPα was recently characterized by Rana et al (29) and these in vitro studies of HepG2 cells showed that C/EBPβ was a positive regulator of the C/EBPα promoter. We have found that the induction of C/EBPβ is delayed in old rats after PH. In both young and old rats the induction of C/EBPβ correlates with the time of C/EBPα mRNA reduction (Fig. 6) suggesting that C/EBPβ might be a negative regulator of C/EBPα expression in vivo. Because the rat C/EBPα promoter contains at least one C/EBP binding site (29) and C/EBPβ has been shown to repress albumin expression during liver regeneration (32), we speculate that the C/EBPβ isoforms can down-regulate C/EBPα via binding to the C/EBPα promoter. During liver regeneration the expression of both C/EBPβ protein isoforms (LIP and LAP) is induced. LIP, the 20kD truncated isoform of C/EBPβ, has been shown to be an inhibitor of full length C/EBP proteins presumably through formation of inactive heterodimers with subsequent occupation of binding sites (24). The way(s) in which LAP and LIP function during regeneration can only be speculated upon,

but it is interesting to note that there are several other biological situations in which expression of C/EBPα and C/EBPβ is inversely related, including the acute phase response (33), TNFα treatment (34) and thermal injury (35). Thus, although C/EBPβ may be considered as a candidate regulatory factor for C/EBPα , its in vivo activity appears to be contradictory to that demonstrated in vitro.

Five proteins showed significant changes in their levels in old rats compared to young: C/EBPβ, C/EBPα, p21, PCNA and cdc2 p34. The p21 protein has been shown to be reduced in the livers of newborn mice deficient for C/EBPα (4). C/EBPα mediated control of the p21 protein in liver does not involve transcriptional regulation of the p21 gene, but rather increases in p21 protein levels. We have shown that C/EBPα interacts with p21 protein in mammalian cells suggesting that this interaction contributes to p21 stability (4). We have also detected alterations in the expression of two other genes that are required at G2/M, PCNA and cdc2 p34. Experiments with cultured cells have shown that PCNA interacts with p21 (15,16) leading to the speculation that alterations of PCNA expression in old rats are dictated by high levels of C/EBPα and/or p21. Cdc2 p34 is a mitotic specific protein that is necessary for transition through the G2/M phase (27,28). Its expression in quiescent rat livers is undetectable. At 48 hrs, the cdc2 p34 level is induced in both young and old rats, but the level of induction is 3-4 fold less in old rats compared to young. In addition, the phosphorylation of cdc2 p34 does not occur in old rats as it does in young animals. The levels of two cell cycle related proteins, PCNA and cdc2 p34, are altered in old rats as would be predicted by the smaller fraction of cells entering the cell cycle. It is also possible that migration of cdc2 p34 to the nucleus is effected in older animals as nuclear extracts were used for these studies. In vitro experiments with cultured HT1 cells growth inhibited by C/EBPα expression also showed

that cdc2 p34 was depressed. Thus, our results demonstrate multiple changes in the expression of the genes that are necessary for G1/S transition in old animals compared to young.

The multitude of changes observed in cell cycle associated proteins would suggest that one or more pathways of the hepatocyte regenerative response are active in young, but not old, animals. Clearly C/EBPα and p21 are expressed in different ways following PH in the two age groups studied and their continued high level of expression is consistent with a delay and reduction in the regenerative response. Other pathways must operate in older animals to "push" the hepatocytes into cell division, because eventually sufficient numbers of cells undergo replication to replace liver mass.

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FIGURE LEGENDS

Figure 1. The proliferative response to partial hepatectomy in old rats is delayed. Livers from young (6-10 mo) and old (24 mo) rats were collected at 6, 24 and 48 hrs after PH (indicated on the left), fixed with formalin and stained with monoclonal antibodies to the S-phase specific protein, PCNA.

Figure 2. C/EBPα protein levels are not reduced in old rats in response to partial hepatectomy. 50 ug of nuclear protein isolated at different times after PH (indicated on the top) were analyzed by Western analysis with antibodies to PCNA, cdk4 and C/EBPα. Western blotting with β-actin was performed after stripping the C/EBPα membrane.

Figure 3. Regenerating livers in old animals have high levels of p21 protein. Nuclear proteins (100 ug) isolated from young and old rats at different time points after PH were analyzed by Western blotting with p21 specific antibodies. Analysis of two sets of hepatectomized animals is shown. Sham operated rats served as the control.

Figure 4. A. Induction of cdc2 p34 protein in old rats is reduced compared to an induction in young animals. **Upper part.** Western analysis with cdc2 p34 antibodies was performed as described above. Laser densitometry analysis and quantitation of cdc2 p34 levels as the ratio to cdk4 indicated that, in young rats, the level of cdc2 p34 at 48 hrs is 3-4 fold higher than in old rats. **Bottom part.** The upper band of cdc2 p34 is the phosphorylated form of cdc2. Nuclear extracts (NE) from young (left) and old (right) rats were treated with increasing amounts (indicated on the top,) alkaline phosphatase

(AP). After the treatment, proteins were analyzed by Western blotting. Positions of phosphorylated (p-cdc2) and unphosphorylated (cdc2) forms are shown on the left. **B.** C/EBPα controls the expression of cdc2 p34 in cultured HT1 cells during growth arrest. HT1 cells contain the C/EBPα gene under Lac-repressor control (10). Expression of C/EBPα in HT1 cells was induced by addition of IPTG (I), proteins were isolated at 1, 2 and 3 days after IPTG addition and analyzed by Western blotting. HT1080 fibrosarcoma cells (that do not have C/EBPα inducible gene) and control HT1 cells treated with Glucose (G), were used as the controls. Upper panel shows C/EBPα induction in response to IPTG. The membrane for cdc2 p34 was probed sequentially with antibodies to cdk4 and β-actin.

Figure 5. Induction of another member of the C/EBP family, C/EBPβ, occurs slightly later in old rats after PH. **A.** Northern analysis of C/EBPα and C/EBPβ mRNA expression in young and old rats in response to PH. Total RNA was isolated from rat livers at different times after PH and analyzed by Northern with specific probes. The membrane was sequentially hybridized with C/EBPα, C/EBPβ and 18S rRNA probes. **B.** Induction of C/EBPβ isoforms, LAP and LIP, in old rats is delayed after PH. Western analysis of nuclear extracts from young and old rats was carried out as described above. Positions of the C/EBPβ isoforms, LAP and LIP, are shown on the left. β-actin staining of the same filter shows relative protein loading. **C.** Quantitation of C/EBPα and C/EBPβ mRNAs levels was done using phosphor imaging. The levels of C/EBPα and C/EBPβ mRNA were calculated as the ratio to 18S rRNA.

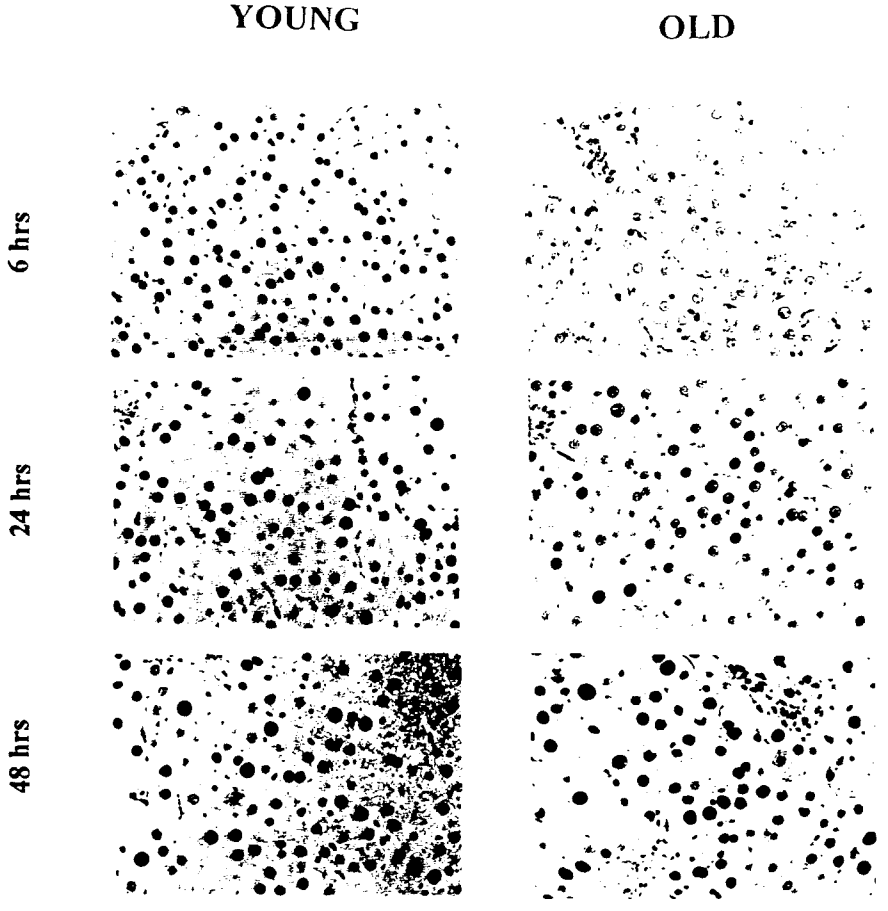


Fig. 1

Fig. 2:

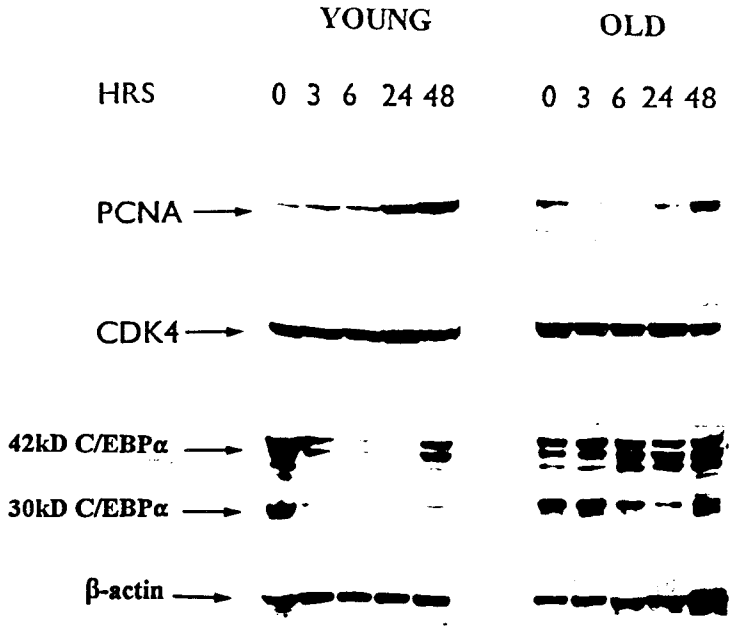
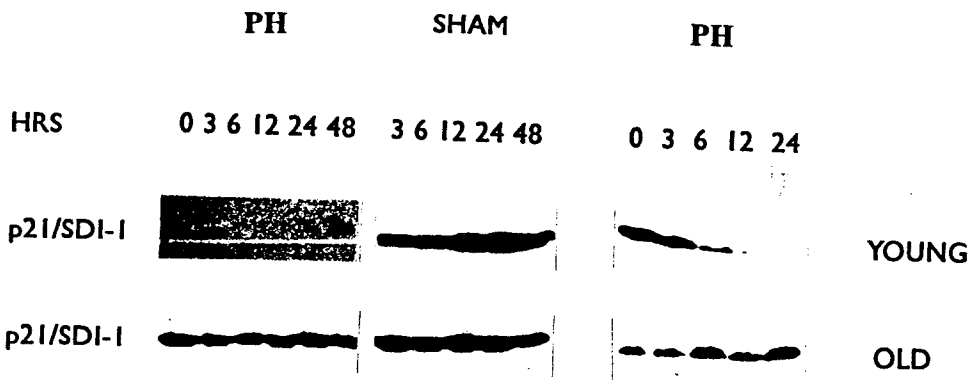
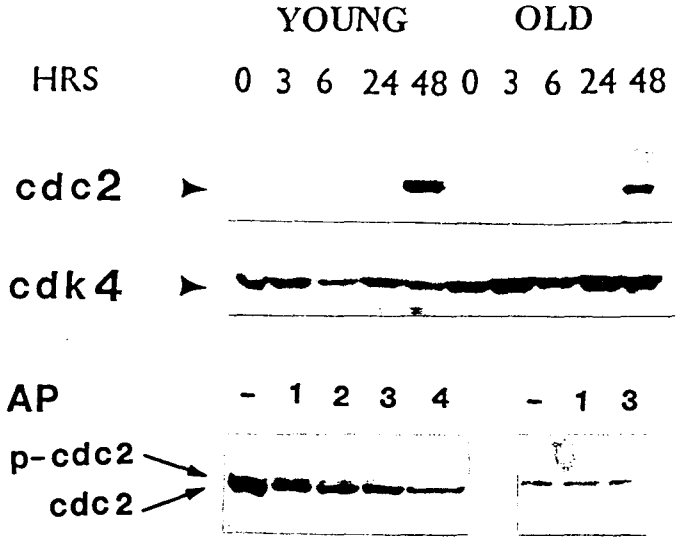

YOUNG
OLD
HRS
0 3 6 24 48
0 3 6 24 48
PCNA
CDK4
42kD C/EBPα
30kD C/EBPα
β-actin

Fig 3.


PH
SHAM
PH
HRS
0 3 6 12 24 48
3 6 12 24 48
0 3 6 12 24
p21/SDI-1
p21/SDI-1
YOUNG
OLD

A


YOUNG
OLD
HRS
0 3 6 24 48 0 3 6 24 48
cdc2
cdk4
AP
- 1 2 3 4 - 1 3
p-cdc2
cdc2

B

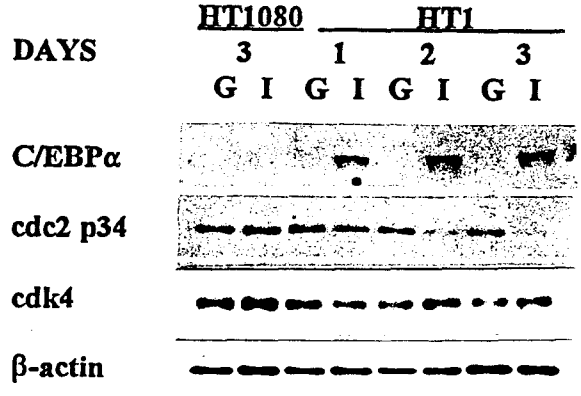
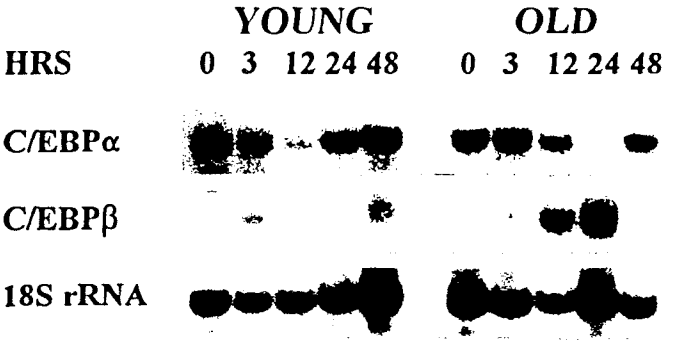

HT1080
HT1
DAYS
3 1 2 3
G I G I G I G I
C/EBPα
cdc2 p34
cdk4
β-actin

Fig. 4

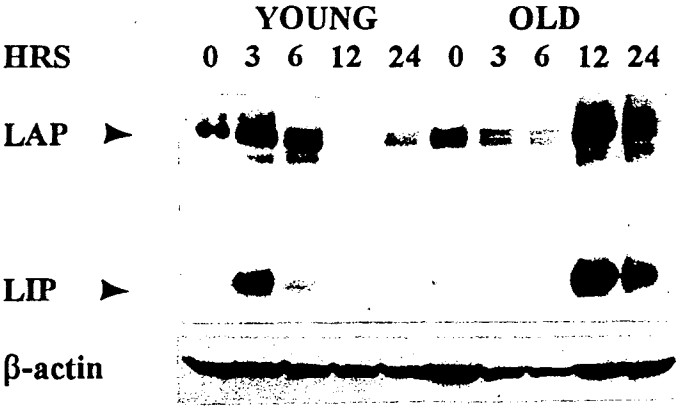
A

NORTHERN



B

WESTERN



C

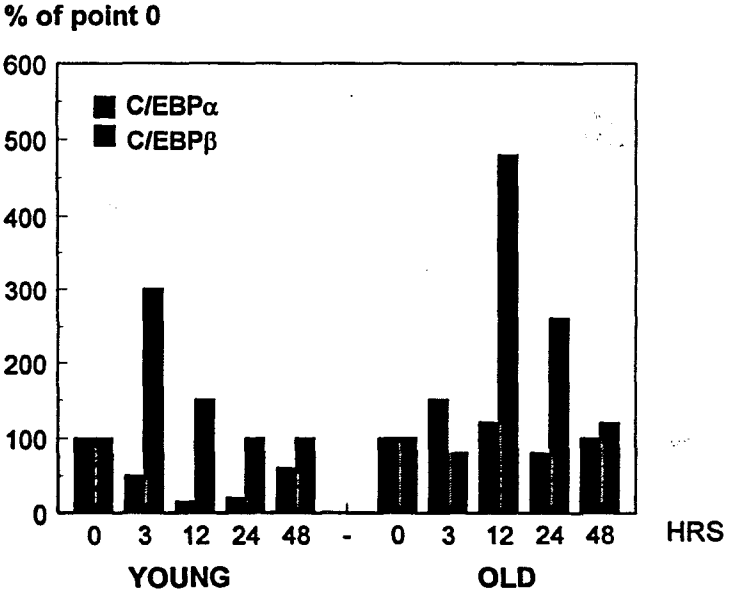


Fig. 5