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Effect of Aging on Regulation of sdi-1 in Rat Hepatocytes

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We examined basal and EGF stimulated DNA synthesis as well as sdi-1 mRNA and protein in primary hepatocyte cultures, and basal levels of sdi-1 mRNA and protein in whole liver homogenates from 6 and 24 month old rats. Since EGF stimulated DNA synthesis decreases with age, it was hypothesized that basal and EGF stimulated levels of sdi-1 mRNA and protein, an inhibitor of DNA synthesis, might increase. Surprisingly, however, both sdi-1 mRNA and protein actually decreased both in cells and homogenates of old rats. These results indicate that the age-related impairment in EGF stimulated DNA synthesis in hepatocytes appears to occur prior to or parallel with sdi-1 expression and cannot be explained on the basis of increased inhibition due to elevated levels of this protein.

Alterations in the regulation of DNA synthesis and cell division represent some of the most important functional manifestations of aging (1). On one hand, impairments in these processes result in deficits in wound healing (2), tissue regeneration (3), and immune response (4). In contrast, uncontrolled cell proliferation leads to neoplasia (5).

Sdi-1 (a.k.a. cip-1, waf-1, p21) is a gene whose product has been shown to inhibit DNA synthesis and prevent both normal and neoplastic cell division (6-9). In addition, its expression increases in late passage human diploid fibroblasts, an *in vitro* model of programmed cessation of DNA synthesis and cell division and/or cellular senescence (6).

We and others have recently developed a complimentary *in vivo* model of cellular aging, primary cultures of rat hepatocytes, which exhibit impaired stimulation of DNA synthesis with increasing donor age (10, 11). EGF, epinephrine and isoproterenol are all less effective in stimulating DNA synthesis in cultures obtained from aged rats than in cells from young animals (10,12). Because growth factors and catecholamine exert their effects through widely different signal transduction pathways (e.g. tyrosine kinases vs. G protein linked receptors) it seems likely that defects in stimulation of DNA synthesis occur at a very fundamental level.

It thus became important to determine whether Sdi-1 increases with *in vivo* aging of hepatocytes in a manner analogous to that occurring during fibroblast passaging *in vitro.* If so, this might provide a generalized mechanism for impairment in DNA synthesis during aging in a variety of cell types. The present study, therefore, compares the levels of both sdi-1 mRNA and protein in primary hepatocyte cultures as well as whole liver homogenates from mature and senescent rats.

MATERIALS AND METHODS

Animals. Male Wistar rats aged 6 and 24 months were obtained from the Gerontology Research Center (National Institute on Aging) colony. These rats have a mean life span (50% mortality) of about 23 months. Rats were

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maintained at 12h light/dark cycles in controlled environment and fed ad libitum on National Institute of Health Purina Chow.

Chemicals. Collagenase (Type 2) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Collagen (Type 1), dexamethasone, aprotinin, trypsin inhibitor and bovine serum albumin were obtained from Sigma Chemical (ST. Louis, MO). EGF was obtained from GIBCO BRL (Gaithersburg, MD). Hanks' balanced salt solution (HBSS) and Williams medium E were obtained from Quality Biological Inc. (Gaithersburg, MD). anti-sdi-1 monoclonal antibody was a gift of Dr. Wade Harper. [Methyl-^{3}H]Thymidine (85 Ci/mmol) was obtained from Amersham Co. (Arlington Heights, IL). A 707 bp mouse cDNA fragment was used as a probe for sdi-1 RNA (13). phcGAP (14), which carries a full-length human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, was obtained from the American Type Culture Collection. All other chemicals used were the highest grade commercially available.

Primary hepatocyte cultures. Rat hepatocytes were isolated by the collagenase perfusion method as described before (10). The isolated cells were suspended at 2.5×10^5 cells/ml in Williams medium E containing 0.1 ug/ml aprotinin and 10^{-9} M dexamethasone supplemented with 5% fetal calf serum (FCS) and plated into 22mm-diameter wells of multiwell culture plates and 10 cm-diameter dishes, which had been coated with calf skin type 1 collagen. Cells were cultured at 37°C under 5% CO_2 in air for 3 h to allow attachment to the dishes, then the medium was replaced with serum-free Williams medium E containing 0.1 ug/ml of aprotinin and 10^{-9} M dexamethasone. Cell viability was determined by trypan blue dye exclusion before plating.

Whole liver homogenates. Rat liver was perfused with 100 ml of EGTA solution containing 0.5mM EGTA, 5mM glucose, 136mM NaCl, 5.3mM KCl, 0.3mM Na_2HPO_4, 0.4mM KH_2PO_4 and 10mM HEPES (pH 7.2) at a rate of 20ml/min, minced on ice immediately after removal from the animal and homogenized with a Kinematic Polytron (30 s at setting 6).

Assessment of DNA synthesis. DNA synthesis was assessed by measuring incorporation of [^{3}H]thymidine into DNA as described before (10). EGF (100ng/ml) was added 20 h after cell inoculation, and then at appropriate times, [^{3}H]thymidine (10 uCi/ml, 85 Ci/mmol) was added. After 2h, the cells were washed twice with phosphate-buffered saline (PBS) and immersed in 1ml of 10% trichloroacetic acid (TCA). The hepatocytes were solubilized by incubation at 37°C for 30 min in 0.5 ml of 1N NaOH, and 100% TCA was added to the solution to a final concentration of 10%. The precipitate was washed twice with 10% TCA and hydrolyzed by heating at 90°C for 15 min in 0.5 ml of 10% TCA. Radioactivity in the hot TCA-soluble fraction was measured. Protein was measured by the method of Lowry et al. (15) using bovine serum albumin as a standard.

Measurement of sdi-1 mRNA. Total cellular RNA was purified from cultured hepatocytes and whole liver homogenate by the method of Chomczynski and Sacchi (16) and quantitated spectrophotometrically. Northern blot analysis was performed by standard methods using 20μg total cellularRNA (17, 18). Radioactive probes were prepared by the method of random priming and blots were hybridized according to standard procedures (17, 18). Membranes were stripped of the labeled probe following manufacturer instructions and rehybridized with GADPH. The membranes were scanned with an Ambis radioanalytic scanning system to quantitate the amount of radioactivity in the individual bands.

Measurement of Sdi-1 protein. Cells were washed once with PBS and lysed in 500ul of Laemmli sample buffer (19). The lysates and whole liver homogenate were normalized to equivalent total cellular protein levels, as determined by Bradford analysis (20), and electrophoresed on 16% polyacrylamide gels. Proteins were transferred to nitrocellulose paper as described by Towbin et al. (21) and immunoblotted with anti-sdi-1 monoclonal antibody and horseradish peroxidase-linked sheep anti-mouse IgG antibody. Immune complexes were visualized using the enhanced chemiluminescence procedure (Amersham Co, Arlington Heights, IL). Quantitation of exposed films was carried out by using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. All values are expressed mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA). Differences between individual age or treatment groups were evaluated using the unpaired two-tailed Student's t test.

RESULTS

Effect of Aging on EGF Stimulated DNA Synthesis

Hepatocyte DNA synthesis in response to EGF was assessed using 2h pulses of [^{3}H]thymidine. We demonstrated previously that [^{3}H]thymidine incorporation in cultured hepatocytes from rats of various ages increased 12h after addition of EGF, reached a peak time at 48h, and then gradually decreased (12). Therefore, we examined [^{3}H]thymidine incorporation 48h after addition of EGF to primary cultures from 6 and 24 month old rats, in order to be certain that DNA synthesis was indeed reduced in the aged hepatocytes under examination. Figure 1 shows that the magnitude of DNA synthesis 48h after addition of EGF in 24 month old rats was reduced about 70% as compared with 6 month old rats.

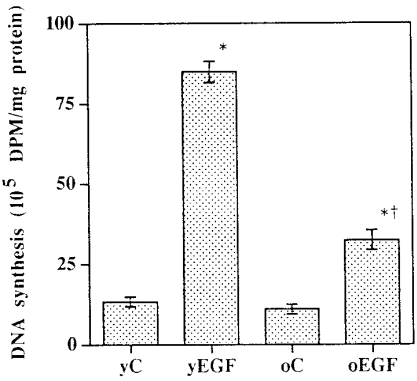

FIG. 1. Effect of aging on EGF stimulated DNA synthesis in primary cultured hepatocytes from 6 and 24 month old rats. EGF(100ng/ml) was added to the culture medium 20h after cell inoculation. Cells were then incubated for 48h and labeled with [^{3}H]thymidine for 2h. Values are expressed as means ± SEM for 4 individual animals. Significance: *P<0.001 compared to unstimulated control. +P<0.001 compared to 6 month old rats.

Expression of sdi-1 mRNA during Aging

Sdi-1 mRNA expression during aging was examined by Northern blot analysis. Twenty micrograms of RNA from primary hepatocyte cultures and whole liver homogenates from 6 and 24 month old rats were sequentially hybridized to ^{32}P-labeled sdi-1 cDNA and GAPDH. The sdi-1 probe hybridized to a 2.1-kb cellular transcript. The expression of sdi-1 mRNA in both cultured hepatocytes and whole liver homogenates was reduced with age, whereas the expression of GAPDH mRNA was roughly equivalent in 6 and 24 month old rats (Fig. 2). The relative amount of sdi-1 mRNA / GAPDH mRNA in cultured hepatocytes and whole liver homogenate from 24 month old rats was reduced about 50 and 20%, respectively, as compared with 6 month old rats.

Expression of sdi-1 Protein during Aging

Sdi-1 protein content during aging was examined by Western blot analysis. Forty micrograms of protein from primary hepatocyte cultures and whole liver homogenates from 6 and 24 month old rats was electrophoresed, transferred to nitrocellulose paper and immunoblotted. The Sdi-1 protein was detected as a 21 kd band. The level of Sdi-1 protein in primary cultures and whole liver homogenates from 24 month old rats was reduced about 69 and 70%, respectively, as compared with 6 month old rats (Fig. 3). Although, large variability between experiments was observed (possibly due to differences in exposure times, washing conditions, etc.), relative age differences were quite reproducible.

Time Course of EGF Stimulated Sdi-1 mRNA Expression during Aging

To determine whether changes in sdi-1 expression during the course of EGF stimulation might contribute to age differences in DNA synthesis, we examined expression of the gene in primary cultures from 6 and 24 month old rats synchronized in G0 by deprivation of serum growth factors for 20h and then stimulated to enter the cell cycle by the addition of EGF. Despite some variability among experiments, EGF increased the sdi-1 GAPDH mRNA ratio in hepatocytes from 6 month old rats slightly at 6h followed by a decline to approximately 70% of basal level at 48h. In contrast, the sdi-1 GAPDH mRNA ratio in hepatocytes from 24 month old rats decreased by 20% at 6h and then remained unchanged (fig. 4). Accordingly, the age × time interaction for the 6 and 24 month old groups was significantly different ($p<0.01$, $F=14.5$).

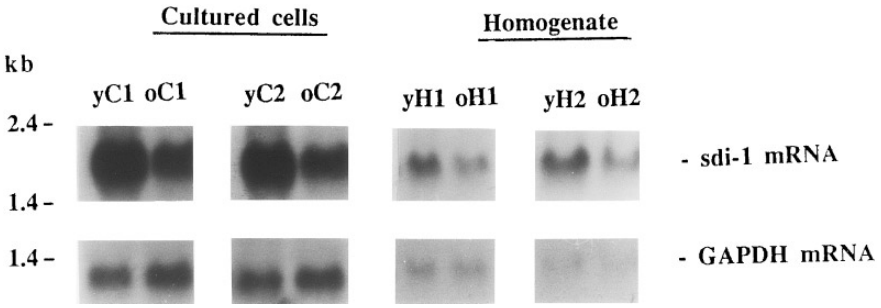

FIG. 2. Northern blot analysis of sdi-1 and GAPDH in primary cultured hepatocytes (C) and whole liver homogenates (H) from 6 (y) and 24 (o) month old rats in 2 representative experiments. Northern blots of 20 micrograms of RNA isolated from primary cultured hepatocytes and whole liver homogenates were probed sequentially with sdi-1 and GAPDH (as a loading control) cDNAs. ODs (in OD units) were obtained from integrated areas on the autoradiographic film: 6 months-173 and 185, 24 months-86 and 122 for sdi-1 in cultured cells in the two experiments. Homogenate values were : 6 months-25 and 28, 24 months-18 and 17, GAPDH values were: 6 months-263 and 239, 24 months-300 and 279 for cultured cells and 6 months-12 and 8, 24 months 11 and 6 for homogenates.

Time Course of EGF Stimulated Sdi-1 Protein Content during Aging

We also examined the levels of the Sdi-1 protein 0, 6 and 48h after the addition of EGF. As demonstrated in Fig 3, basal Sdi-1 content was significantly reduced by 69% with age ($p<0.01$). The levels of Sdi-1 protein in hepatocytes from 6 month old rats increased by 40% at 6h ($p<0.05$) and then decreased to 80% of basal level at 48h. In contrast the levels of sdi-1 protein in hepatocytes from 24 month old rats remained unchanged (fig. 5). Preparations from respective age/treatment groups shown here are from additional experiments to those in Fig. 3, were all processed simultaneously, and yielded very similar values.

DISCUSSION

Present studies have demonstrated that basal and EGF stimulated levels of sdi-1 mRNA and protein in primary cultured hepatocytes are reduced with age. Thus, increased sdi-1 content cannot explain age-related decrease in EGF stimulated DNA synthesis. Furthermore, the levels of sdi-1 mRNA and protein in whole liver homogenate are also reduced with age, suggesting that data from cell cultures are not preparation artifacts. We previously demonstrated that stimulation of hepatocyte DNA synthesis by both beta-adrenergic agonists and EGF declined markedly during aging (10,12). These responses did not appear to be the consequence of

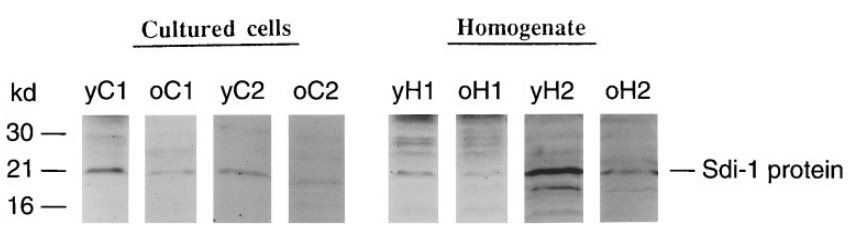

FIG. 3. Western blot analysis of Sdi-1 protein in primary cultured hepatocytes (C) and whole liver homogenates (H) from 6 (y)and 24 (o) month old rats in 2 representative experiments. Forty micrograms of protein from primary cultured hepatocytes and whole liver homogenate was electrophoresed, transferred to nitrocellulose paper and immunoblotted with anti-Sdi-1 monoclonal antibody and horseradish peroxidase-linked sheep anti-mouse IgG antibody. ODs (in OD units) were: 6 months-213 and 120, 24 months-60 and 42 for cultured cells in the two experiments. Homogenate values were : 6 months-114 and 828, 24 months-54 and 225.

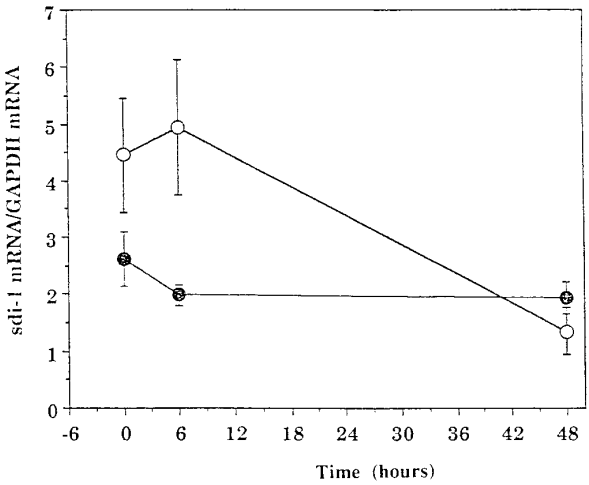

FIG. 4. Time course of EGF stimulated sdi-1 mRNA expression. Hepatocytes from 6 (○) and 24 (●) month old rats were seeded into 10 cm-diameter dishes in medium containing 5% FCS. Three hours later, the medium was replaced with serum-free medium. Twenty hours later, the cultures were stimulated with fresh medium containing 100 ng/ml EGF. RNA was extracted at the indicated times and subjected to Northern blot analysis. The membrane was probed sequentially with sdi-1 and GAPDH ^{32}P-labeled cDNAs. Quantitation was performed as described and the ratio of sdi-1/GAPHD intensity plotted for the given times after EGF stimulation.

receptor loss (12). The fact that the signal transduction pathways for these agents are quite different suggests that age changes occur at a very fundamental level.

In addition, we previously reported that the expression of sdi-1 increased 10- to 20-fold in late compared with early passage human diploid fibroblasts and the increase in mRNA closely paralleled the onset of the senescent phenotype and loss of cell proliferation. Following serum stimulation, sdi-1 mRNA levels in young (early passage) cells were initially increased, but

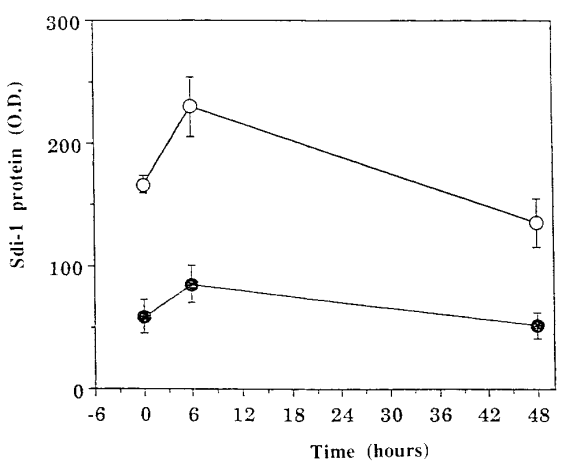

FIG. 5. Time course of EGF stimulated Sdi-1 protein level. Hepatocytes from 6 (○) and 24 (●) month old rats were seeded into 10 cm-diameter dishes in medium containing 5% FCS. Three hours later, the medium was replaced with serum-free medium. Twenty hours later, the cultures were stimulated with fresh medium containing 100ng/ml EGF. Protein was extracted at the indicated times and subjected to Western blot analysis. Quantitation was performed as described and optical density O.D.) plotted for the given times after EGF stimulation.

then declined to low levels just prior to the entry of the cells into S phase. In contrast, RNA levels of sdi-1 in senescent (late passage) cells failed to decline, suggesting a role for this gene in maintaining the senescent phenotype.

Present results suggest that the mechanism of age-related impairment in stimulated DNA synthesis in primary cultures of rat hepatocytes is quite different from that in cultured fibroblasts. These findings further suggest that the age-related impairment in stimulated DNA synthesis in hepatocytes appears to occur prior to or parallel with sdi-1 expression. Possible candidate events include signal transduction through protein kinases (22), transcription factors (23), and/or DNA polymerase alpha activation (24, 25). Further studies are necessary to reveal the precise mechanism of age related impairment in stimulated DNA synthesis in hepatocytes.

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