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EFFECT OF AGING ON EGF STIMULATED DNA SYNTHESIS AND EGF RECEPTOR LEVELS IN PRIMARY CULTURED RAT HEPATOCYTES

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Summary: Epidermal growth factor (EGF) stimulated DNA synthesis and EGF receptor levels were examined in primary cultured hepatocytes from 6, 12 and 24 month old rats. EGF stimulated DNA synthesis began after 12h and reached a peak at 48h. Although no age difference was seen in the time course of DNA synthesis, the magnitude of synthesis at the peak time in 12 and 24 month old rat hepatocytes was reduced approximately 50 and 70%, as compared to that at 6 months. Hepatocyte EGF receptors exhibited no age difference in the equilibrium dissociation constant (Kd) or the density (Bmax). These results indicate that EGF stimulated DNA synthesis in rat hepatocytes declines with age, and that this reduction is not due to decreased receptor density or specific binding affinity.

Impaired ability to stimulate DNA synthesis and cell division is characteristic of many cell populations during aging (1). Experimental models include lymphocytes, fibroblasts, epithelial and parenchymal cells from blood, lung, liver, salivary glands and other tissues (2-7). For example, studies in vivo demonstrated long ago that DNA synthesis following partial hepatectomy was delayed and decreased in magnitude as rats aged (6). More recently, we have observed similar age-associated reductions in responsiveness of rat hepatocyte primary cultures to catecholamines in vitro (8). This response appears to be mediated through the beta-adrenergic receptor system (8). Although numerous laboratories have examined beta-adrenergic responsiveness in many tissues, including liver, during aging (9-11), localization of the precise signal transduction components/events responsible for the age changes in this case have not yet been achieved.

Nevertheless, the fact that stimulated DNA synthesis is compromised during aging in so many systems does suggest that an impairment may occur at a very fundamental level. In order to examine this possibility, we took advantage of the fact that hepatocyte DNA synthesis can also be stimulated by EGF in vitro. This effect is mediated through a different signal transduction pathway than the beta-adrenergic response and involves autophosphorylation of the receptor by an endogenous protein kinase and possibly specific gene transcription (e.g. c-myc and c-fos) (12,

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13). Present results indicate that EGF stimulated DNA synthesis in rat hepatocytes in vitro is also reduced during aging and that this response is completely blocked by aphidicoline. Thus (a) fundamental alteration(s) may occur in the ability to regulate DNA replication during hepatocyte aging.

MATERIALS AND METHODS

Animals. Male Wister rats aged 6, 12 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 maintained at 12h day/dark cycles in controlled environment and fed *ad libitum* on National Institute of Health Purina Chow.

Chemicals. Collagenase (type IV), collagen (type I), 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 NIH media unit. [methyl-^{3}H]-thymidine (85 Ci/mmol) and ^{125}I-EGF (100 μCi/μg) were obtained from Amersham Co. (Arlington Heights, IL). All other chemicals used were the highest grade commercially available.

Isolation and culture of hepatocytes. Rat hepatocytes were isolated by the collagenase perfusion method as described before (8). The isolated cells were suspended at 2.5×10^5 cells/ml in Williams medium E containing 0.1μg/ml aprotinin and 10^{-9}M dexamethasone supplemented with 5% fetal calf serum (FCS) and plated into 22 mm-diameter wells of Coaster multiwell culture plates, which had been coated with rat type I collagen. Cells were cultured at 37°C under 5% CO_2 in air for 3h to allow attachment to the wells, then the medium was replaced with serum-free Williams medium E containing 0.1μg/ml of aprotinin and 10^{-9}M dexamethasone. Cell viability was determined by trypan blue dye exclusion before plating.

Assay of DNA synthesis. DNA synthesis was assayed by measuring incorporation of [^{3}H]thymidine into DNA as described before (8). EGF was added 20h after cell inoculation, and then at appropriate times, [^{3}H]thymidine (10 μCi/ml, 85 Ci/mmol) was added. After 3h, 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 30min in 0.5ml 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 15min in 0.5ml of 10% TCA. Radioactivity in the hot TCA-soluble fraction was measured. EGF stimulated DNA synthesis activity (dpm/mg protein) was calculated by subtracting the values of non-stimulated controls. Protein was measured by the method of Lowry et al. (14) using bovine serum albumin as a standard.

^{125}I-EGF binding assay. ^{125}I-EGF binding to hepatocytes in primary culture was performed as described by Cruise et al. (15) with minor modifications. Hepatocytes were washed once with ice-cold binding medium (Williams medium E containing 1mg/ml bovine serum albumin and 25mM HEPES, pH 7.4) and preincubated at 4 °C for 30min in binding medium. Cells were then incubated at 4 °C for 2h in binding medium containing various amount of ^{125}I-EGF. Two hours of incubation at 4 °C is sufficient to reach steady-state binding conditions for ^{125}I-EGF in primary cultured hepatocytes (16). The cells were then washed rapidly three times with ice-cold binding medium. The total cell-associated radioactivity was determined by solubilization of the cells in 0.5ml of 1N NaOH. The samples were counted in a ICN gamma counter (10/600 PLUS). Nonspecific binding was defined as the radioactivity not displaced in the presence of the 1000-fold excess of nonradioactive EGF and was typically 5% of the total binding. The data were analyzed by computer using the EBDA and LIGAND programs (BIOSOFT, UK).

Statistical analysis. All values are expressed mean±SEM. Statistical analysis between the 3 age groups was performed by one-way analysis of variance (ANOVA). Differences between 2 age 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, and the time course determined in primary cultures from 6, 12 and 24 month old rats is shown in Fig. 1. [^{3}H]thymidine incorporation increased 12h after addition of EGF, reached a peak time at 48h and then gradually decreased. There were no difference in the onset and peak time of DNA synthesis during aging. However, the magnitude of DNA synthesis at the peak time in 12 and 24 month old rats was reduced about 50 and 70%, respectively, as compared with 6 month old rats. EGF stimulated DNA synthesis in hepatocytes from all age groups (including 24 month old rats) was significantly increased over baseline levels ($p<0.001$).

In order to determine whether the observed stimulation of DNA synthesis resulted from replication or from a repair process, we examined the inhibitory effect of aphidicoline, which is a specific inhibitor of the replicative DNA polymerase α (17). The addition of 20ng/ml of aphidicoline to cultured hepatocytes from 6 and 24 month old rats completely abolished EGF stimulated [^{3}H]thymidine incorporation (from 78.2±7.7 to 2.6±0.1 and from 26.8±2.3 to 3.8±0.2

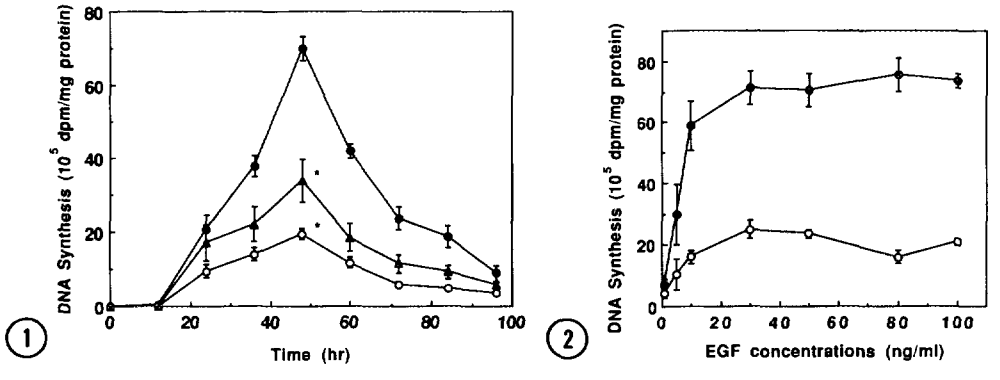

Figure 1. Time course of EGF stimulated DNA synthesis in hepatocytes from 6, 12 and 24 month-old rats. EGF (100ng/ml) was added to the culture medium 20h after cell inoculation. Cells were then incubated and labeled with [^{3}H]thymidine for 2h at the indicated time. DNA synthesis was calculated by subtracting unstimulated values at each time point. Unstimulated control DNA synthesis at a peak time of 6, 12 and 24 month old rats were 15.4±0.4, 15.8±1.4 and 10.0±1.8 x10^5 dpm/mg protein, respectively. Each point represents the mean±SEM of 6 to 7 individual animals aged 6 (●), 12(▲) or 24 (○) months. Significance: * $p<0.001$ compared to 6 month old rats.

Figure 2. Dose-response curve of EGF stimulated DNA synthesis in hepatocytes from 6 and 24 month old rats. EGF was added to the culture medium 20h after cell inoculation. Cells were then incubated for 48h and labeled with [^{3}H]thymidine for 2h. DNA synthesis was calculated by subtracting unstimulated values.Unstimulated controls DNA synthesis of 6 month old rats and 24 month old rats were 12.1±0.5 and 8.9±0.8 x10^5 dpm/mg protein, respectively. Each point represents the mean±SEM of 6 determinations. (●): 6 month old and (○): 24 month old.

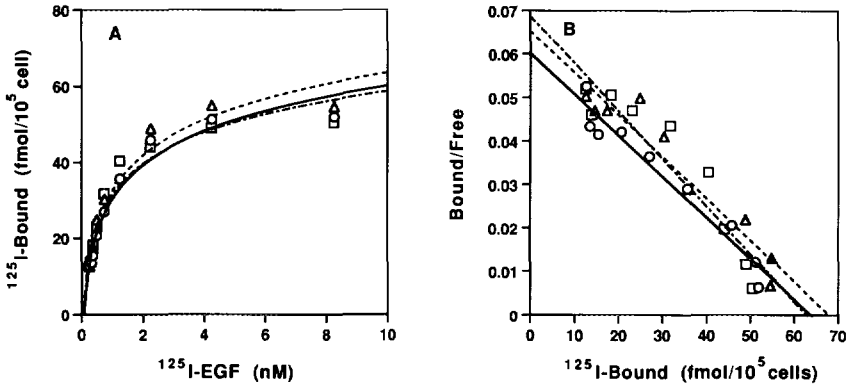

Figure 3. (A) Saturation curve and (B) Scatchard plot of ^{125}I-EGF binding to hepatocytes from 6, 12 and 24 month old rats. Data are from a representative experiment of each age group, performed with triplicate cultures. All saturation curves were adequately modeled to a single class of sites. (○): 6 month old, (□): 12 month old and (Δ): 24 month old.

x10^5 dpm/mg protein in 6 and 24 month old rats, respectively) without any effect on cell viability, as determined by trypan blue dye exclusion (data not shown).

Concentration dependence of EGF stimulated DNA synthesis: Dose-response curves for EGF stimulated DNA synthesis in hepatocytes from 6 and 24 month old rats are shown in Fig. 2. The exposure to EGF of hepatocytes from 6 month old rats resulted in a concentration-dependent stimulation of DNA synthesis (1-30ng/ml), and the response was maximum at concentrations over 30ng/ml. In hepatocytes from 24 month old rats, the concentration dependence was similar to that at 6 months, but the peak magnitude of DNA synthesis was significantly lower than that from 6 month old rats.

Effect of aging on hepatocyte EGF receptor levels: The decreased EGF stimulated DNA synthesis in hepatocytes from 12 and 24 month old rats may be associated with an alteration in cell surface receptors. To assess this possibility, we measured receptor density and specific binding affinity using ^{125}I-EGF. A representative example of Scatchard plot of the equilibrium binding of ^{125}I-EGF to hepatocytes from 6, 12 and 24 month old rats is shown in Fig. 3 and the determination of the equilibrium dissociation constant (Kd) and the receptor density (Bmax) are summarized in Table 1. There were no significant difference in receptor density and specific binding affinity during aging.

Table 1. Summary of specific ^{125}I-EGF binding to hepatocytes from 6, 12 and 24 month old rats

	EGF binding	
Age (months)	Kd (nM)	Bmax (fmol/10^5 cells)
6	1.05 ± 0.13	62.9 ± 2.3
12	1.03 ± 0.09	69.3 ± 6.8
24	1.03 ± 0.19	64.5 ± 2.0

Values are expressed as mean ± SEM for 4 individual animals.

DISCUSSION

The present findings indicate that EGF stimulated DNA synthesis in primary cultured hepatocytes declines with age, and that this reduction is not due to decreased density or specific binding affinity. These findings confirm and extend our previous observation of decreased hepatocyte DNA synthesis mediated through the beta-adrenergic receptor system during aging (8).

It is well known that EGF can induce many cells including primary cultured hepatocytes to enter the replicative cycle (12). In the present study, [^{3}H]thymidine incorporation was used to measure DNA replication. Indeed, [^{3}H]thymidine incorporation was completely abolished by aphidicoline, which is a specific inhibitor of the replicative DNA polymerase α. The time course of DNA synthesis after exposure to EGF revealed no age difference in onset or peak time, although the magnitude of DNA synthesis was significantly decreased with age. In an in vivo experiment, Bucher et al. (6) reported that the initiation was delayed and the maximum DNA synthesis response decreased with age in regenerating rat liver following partial hepatectomy. The alteration in initiation time as well as maximum response may be related to differences between in vivo and in vitro studies, as discussed in our previous report (8).

Since the binding of EGF to cell surface receptors is an initial event leading to DNA synthesis, it is possible that reduced DNA synthesis is due to decreased EGF receptor binding. To test this possibility, we examined receptor binding directly. No difference in EGF receptor density or specific binding affinity occurred during aging. Similar findings have been reported for in vitro aging using WI-38 cells. EGF binding ability remained essentially unchanged throughout the life span, although EGF stimulated DNA synthesis was reduced in senescent cells (18). Thus, alterations in EGF stimulated DNA synthesis may occur at a post receptor level.

One of the earliest signals after ligand binding is the autophosphorylation of the EGF receptor following activation of the protein kinase which is integrated in the receptor molecule (19). It is not known at present whether the any age differences in such early signal transduction occur, but it is clear that this pathway is quite different from beta-adrenergic receptor system which utilizes G protein coupling, activation of adenylate cyclase etc. (13). It might, therefore, be hypothesized that age-related alterations occur in the ability to regulate DNA replication at a very basic level; possibly in the process of polymerase α activation.

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