Reduced DNA Synthesis in Primary Cultures of Hepatocytes From Old Mice is Restored by Thymus Grafts

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We previously observed in vivo that a neonatal thymus grafted into old mice can correct age-related changes such as occurrence of hepatocyte tetraploid nuclei and impaired isoproterenol-induced DNA synthesis in submandibular glands. The aim of the present paper was to study the influence of age and thymus on basal and $\beta$-adrenergic-stimulated DNA synthesis using primary cultures of mouse hepatocytes. In the absence of any adrenergic agents, cells from young mice show peak DNA synthesis between 36 and 48 h; old mice show a similar time course, but the peak is significantly reduced statistically. The main result is represented by the behavior of hepatocytes from old thymus-grafted mice, which recover the levels of $[^{3}H]$-thymidine incorporation toward young-like values. Grafted animals also show a correction of total DNA content that is increased in old mice. The addition of isoproterenol does not modify the DNA synthetic pattern, whereas the antagonist propranolol causes a slight but statistically significant decrease.

AGING is accompanied by many progressive changes involving different tissues and functions. Some of these modifications are not definitive; for instance, we have previously demonstrated that a neonatal thymus graft exerts restoring actions on various functions found altered in old mice. An age-related increase in hepatocyte nuclear volume and the occurrence of tetraploid nuclei have been demonstrated in the liver; these modifications can be corrected by grafting a neonatal thymus into old mice (1,2). Age-related alterations in functions such as DNA synthesis, density, and regulation of adrenergic receptors observed in brain and submandibular glands, as well as of some hormonal levels, are also corrected by thymus grafts (3–5).

In particular, we studied in vivo stimulation of DNA synthesis induced in mouse submandibular glands by a single injection of the $\beta$-adrenergic agonist isoproterenol (IPR). This response becomes progressively impaired with advancing age (4) as previously observed in rats (6). Neonatal thymus was found capable of restoring a young-like pattern of DNA synthesis when implanted into old animals 1 month before the assay (3). Alterations of IPR responsiveness have also been found in in vitro experiments. Hepatocytes from old rat liver have shown decreased IPR-induced DNA synthesis when compared to cells from young donors (7).

These data prompted us to examine the influence of age on basal and $\beta$-adrenergic-stimulated DNA synthesis and the eventual restoring action of the thymus in mouse hepatocyte cultures. Thus, cultures of hepatocytes from young, old, and old thymus-grafted mice were studied for their ability to synthesize DNA either in the presence or absence of the $\beta$-agonist IPR or the $\beta$-antagonist propranolol (PROP).

Materials and Methods

Material

Materials were from Gibco [Paisley, U.K.; Medium 199, newborn calf serum (NCS), and HEPES], Boehringer Mannheim (Indianapolis, IN; collagenase B, trypsin inhibitor, kanamycin), Aldrich (Milwaukee, WI; isoproterenol); all other products were from Sigma (St. Louis, MO).

Animals

Animals were taken from the BALB/c-nu mouse colony of Gerontologic Research Department, Italian National Research Centers on Aging (Ancona, Italy). The term “nu” refers to the recessive nude mutation introduced into inbred BALB/c mice by crossing them with nude mutants. Young mice were 3–4 months old, whereas old and thymus-grafted old mice were used at 23–25 months of age. Thymus grafts were performed by implanting neonatal thymus under the kidney capsule in Balb/c mice used at 22–23 months of age. They were used 30–90 days after grafting, employing littersmates as old controls. It is worth noting that previous studies have shown that thymus is effective from 1 to 4 months. Grafted mice, in which the implanted thymus was not found at the time of sacrifice, were excluded (two animals out of nine); in early experiments this type of animal served as a suitable control in place of sham-operated ones (3). Other early findings showed that the implantation of dissociated thymocytes or splenic lymphocytes was not effective (8). Experiments were performed by repeating various trials with at least one animal per variable group each time.
Isolation and Culture of Hepatocytes

Hepatocytes were isolated by liver perfusion with collagenase (9,10). Mice were killed by cervical dislocation, and the liver was first perfused with EGTA solution [0.5 mM EGTA, 5.5 mM glucose, 142 mM NaCl, 6.7 mM KCl, 10 mM HEPES (pH 7.6), 100 mM insulin, 0.1 mg/ml kanamycin] and then with collagenase solution [80 units/ml collagenase, 5 mM CaCl\(_2\), 117 mM NaCl, 6.7 mM KCl, 30 mM HEPES (pH 7.4), 5.5 mM glucose, 0.005% trypsin inhibitor, 0.1 mg/ml kanamycin, 1% bovine serum albumin (BSA)] at 5.5 ml/min. The excised liver was dispersed in Medium 199 containing 10% NCS, 0.1 mg/ml kanamycin, and 10 \(\mu\)M dexamethasone (incubation medium) and filtered through nylon mesh. Thereafter, the suspension was centrifuged and washed four times at 20 \(\times\) g for 3 min to remove nonparenchymal cells. The final pellet was suspended in incubation medium and plated (1 ml) into 22-mm-diameter wells of multiwell culture plates at a concentration of 150,000 viable cells per well. Mouse cell viability, determined by trypan blue exclusion, was more than 50% (up to 72%), and no statistically significant differences were found among the three animal models studied (10). Preliminary experiments showed no influence of these viability differences on our results, in agreement with other reports (11). In each experiment, at least two different cell concentrations were used at 75\(\times\)10\(^3\) (\(O\)), 150\(\times\)10\(^3\) (\(A\)), and 200\(\times\)10\(^3\) (\(A\)) cells per well. Increasing cell concentration [\(\mu\)Ci/ml, 5 Ci/mmol] for 2 h. Thereafter, cells were washed twice with phosphate-buffered saline (PBS), immersed in 1 ml of 10% trichloroacetic acid (TCA), and suspended by incubation at 37\(^\circ\)C for 30 min in 0.5 ml 1 M NaOH. Finally, 100% TCA was added and maintained at 4\(^\circ\)C overnight. The precipitate was washed twice with 10% TCA and hydrolyzed by heating at 90\(^\circ\)C for 15 min in 0.75 ml of 10% TCA. Radioactivity was measured in one sample of this hot TCA-soluble fraction. Total DNA content was assayed in every pool of triplicate samples by the method of Dische as modified by Burton (13). Counts (dpm) were corrected for protein content, which was measured on the precipitate suspended in 1 ml 1 M NaOH by the method of Lowry et al. (14) using two different concentrations and BSA as a standard.

**Influence of Cell Concentration on DNA Synthesis**

The rate of DNA synthesis in hepatocyte cultures is inversely proportional to cell concentration. An example of the relationship is reported in Figure 1, which compares DNA synthesis occurring at four cell concentrations, with all cells coming from the same young mouse. Similar results were obtained from old and thymus-grafted old mice (data not shown). When not otherwise specified, results reported in the present paper refer to experiments performed with 150,000 viable cells per well (though every experiment was performed with at least two different cell concentrations: 150,000 and 100,000 or 200,000 cells/well). The choice of 150,000 cells/well allowed us to have a sufficient amount of material for the DNA assay.

**Autoradiography**

Autoradiography was performed on IPR-treated and control samples from young mice. Hepatocytes (2.5 ml of viable cell suspension) were plated at a concentration of 100,000 cells/ml into 35-mm-diameter wells containing a coverslip on their bottom. \([\text{H}]\text{TdR}\) (6 \(\mu\)Ci/ml, 5 Ci/mmol) was first added to the cultures at the same time of IPR addition, and then at 24 and 36 h to obtain the cumulative labeling index. At the end of the incubation time (36 or 48 h) wells were rinsed twice with PBS and cells were fixed in methanol for 15 min. Subsequently, the coverslips were removed and placed on slides. A "Tritium Sensitive Storage" screen suitable to be analyzed by the Cyclone Storage Phosphor System (Packard, Meriden, CT) was exposed to samples for 4 days.

**Statistical Analysis**

Results are expressed as the mean ± SEM. The statistical significance of the influence of animal model and time of culture on hepatocyte \([\text{H}]\text{TdR}\) uptake was determined by using analysis of variance with replication: time of culture
Results

Effect of Age on NCS-Stimulated DNA Synthesis in Mouse Hepatocytes

Results of experiments performed on hepatocytes from young and old mice are reported in Figure 2. DNA synthesis in young animals in the absence of specific stimulation (other than NCS) shows rather low values at early times followed by an increase, which reaches a peak between 36 and 48 h. Old mice show a similar kinetics, which, however, is significantly lower when compared with results from young animals. The interaction term and the main effects of time of culture and animal model on DNA synthesis are statistically significant (see the legend to Figure 2).

Effect of IPR on DNA Synthesis in Hepatocytes of Young and Old Mice

Findings obtained after exposure to IPR are reported in Figure 3. Hepatocytes from young animals cultured in the presence of $2 \times 10^{-5}$, $2 \times 10^{-4}$, $2 \times 10^{-3}$ M IPR do not show any statistically significant changes in $[^3H]$TdR uptake when compared to untreated cells. The slight decreasing trend observed after IPR stimulation, instead of showing the expected increase, could suggest an inhibitory influence of the drug. It is useful to know that the power of the test (significance level $p < .05$) was used for multiple comparison of the differences at various times of culture. Because of the tendency of increasing variability with the magnitude of the observations, we used logarithmic transformation in the analysis of data reported in Figures 2 and 4.

Restoring Influence of Thymic Grafts on Impaired DNA Synthesis in Cells of Old Mice

Figure 4 shows the pattern of DNA synthesis of hepatocytes from young, old and thymus-grafted old mice when

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>C</th>
<th>IPR</th>
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<tbody>
<tr>
<td>36</td>
<td>31.8 ± 1.8</td>
<td>28.4 ± 2.5</td>
</tr>
<tr>
<td>48</td>
<td>57.8 ± 2.6</td>
<td>55.9 ± 1.9</td>
</tr>
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Notes: C, control; IPR, culture cells incubated with $2 \times 10^{-4}$ M (±)-isoproterenol. Values are representative of means ± SEM from four animals at 3 months of age.
cells were cultivated in presence of NCS alone. These results confirm the lower peak of DNA synthesis in cultures from old animals as previously described; in addition, these data demonstrate a restoring action of the thymic graft. In Figure 4A the time course of [3H]Tdr incorporation is reported at a concentration of 150,000 cells per well. Grafted mouse cultures show statistically significant differences from those of old mice, with values close to the ones of young animals, except at 72 h. Figure 4B, referring to 100,000 cells per well, presents similar qualitative patterns of DNA synthesis for the three animal models, although with higher absolute values of [3H]Tdr uptake.

Caution must be used in interpreting results on the comparison between young and thymus-grafted old mice derived data: the lack of statistically significant differences in all data except at 72 h does not mean that thymus recovers most of the altered kinetics of DNA synthesis to the level of young mice. In fact, the power of the test (significance level p < 0.05) to detect a 30% difference is low, ranging from 0.3 to 0.6, according to the time chosen for the comparison. It is worth noting, however, that the key point in our reversibility experiments is the finding that in old thymus-grafted animals that the parameter values were statistically significantly different from those observed in old untreated ones.

A similar trend is also suggested by data from the example shown in Figure 5. Although the young animals present sharp increases in [3H]Tdr uptake at the lower concentrations, the influence of the thymus in increasing DNA synthesis with respect to the low levels of the untreated old animals can be observed at every cell concentration used.

**Table 2. Propranolol Inhibition of [3H]Tdr Incorporation in Hepatocytes From Young Mice**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>DNA Synthesis 10^6 dpm/mg Protein ± SEM</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>139 ± 21**</td>
<td>41</td>
</tr>
<tr>
<td>PROP</td>
<td>82 ± 4</td>
<td>29</td>
</tr>
</tbody>
</table>

Notes: C, control; PROP, culture cells incubated with 2 × 10^-5 M (±)-propranolol; experiment performed on five mice at 3 months of age. Analysis of variance with replication shows a statistically significant influence of PROP on DNA synthesis.

*p = .036; **p = .018.

**Figure 4.** A neonatal thymus grafted into old recipients recovers DNA synthesis toward young-like levels. Young (●), old (○) and thymus-grafted old (old+T) (●) mouse hepatocytes were cultivated in the presence of heat-inactivated NCS only. (A) Data represent means from eight young, eight old, and seven old+T mice. Data at 42 h (young) and 54 h (old and old+T) are missing due to technical reasons. Results of statistical analysis (analysis of variance with replication: time of culture = within-subjects factor, 7 levels; animal model = between-subjects factor, 3 levels) show that (i) the between-subjects main effect of animal model (p < .0001), (ii) the interaction term time × animal (p < .005), and (iii) the within-subjects main effect of time of culture (p < .0001) are statistically significant. Some interest may also reside in point-to-point differences from different animal models checked at various times. Results (Student-Newman-Keuls’ test; level of significance: p < .05) show that point-to-point differences from young and old cultures are statistically significant from 24 h values; and differences from old and old+T are significant from 24 h values except that at 72 h; young and old+T values differ significantly only at 72 h. (B) Data from part of the same animals used in experiments shown in A; results are referred to hepatocytes plated at a concentration of 100,000 cells/well. Data come from four mice only and represent an example showing that even at a lower concentration the general patterns of DNA synthesis in the three animal models are roughly the same. Results of statistical analysis performed on such a small number of animals (analysis of variance with replication: time of culture = within-subjects factor, 8 levels; animal model = between-subjects factor, 3 levels) show that the between-subjects main effect of animal model (p = .008) and the within-subjects main effect of time of culture (p < .0001) but not the interaction term time × animal (p = .069; Pillais’ test) are statistically significant.
DISCUSSION

The main task of the paper was to study the influence of age on β-adrenergic-stimulated DNA synthesis in mouse hepatocyte cultures and the eventual effects of thymic grafts. Although no stimulation has been obtained in hepatocyte cultures from the animals studied, interesting results have also been obtained in the absence of any specific stimulating drug.

The pattern of DNA synthesis found in mouse hepatocyte cultures in the absence of exogeneous stimulating factors except heat-treated NCS is rather different from that observed in rats under similar experimental conditions. At peak time, [³H]TdR incorporation in mouse hepatocytes increases up to about 100-fold over initial levels whereas that from rat hepatocytes increases less than 10-fold (7). Even more striking is the difference following exposure to IPR. In rat hepatocytes a clear increase of DNA synthesis over basal level can be demonstrated (7), whereas mouse hepatocytes do not show any changes with respect to unstimulated levels; on the contrary, there seems to be a slight decreasing trend of [³H]TdR incorporation. The above-mentioned findings and the results from autoradiographic experiments, showing the same cumulative labeling index for IPR-treated and untreated hepatocyte cultures, suggest that the heat-inactivated NCS would contain some murine stimulating agents, although the effects of different physiological environments cannot be excluded. As an example, normal calf serum does contain a hepatocyte growth stimulating factor (HGSF) acting on BALB/c mouse hepatocytes; however, because HGSF is unstable upon heat treatment (15), it cannot help us to explain these data.

The lack of responsiveness of cultures to IPR cannot allow us to speculate that β-adrenergic system is not involved in mouse hepatocyte DNA synthesis. PROP is actually capable of inhibiting [³H]TdR incorporation in a slightly but statistically significant manner. This behavior reflects the actual complexity of the adrenergic control on hepatocyte growth and division including dose and time dependence. In fact, it has been demonstrated that adrenergic stimulation mediated by both α (12,16) and β (17) receptors does affect hepatocyte DNA synthesis in opposite ways depending upon time of culture, such behavior probably reflecting receptor changes during cell cycle (18).

The more striking results of the present paper, however, are those concerning the restoring action of the thymus exerted on the altered kinetics of DNA synthesis observed in old mice, as shown in Figure 4. Hepatocytes obtained from old animals show an increase in DNA synthesis activity in the absence of adrenergic stimuli; however, a clear impairment in DNA synthetic rate has been observed with respect to cells derived from young mice. It remains to be demonstrated whether the impairments observed in old animals are also linked to altered characteristics of receptors for hormones and growth factors, a type of finding frequently observed in various tissues during aging (19). On the contrary, the present results clearly demonstrate that the thymus corrects the age-related impairments. In fact, DNA synthesis in hepatocyte cultures from thymus-grafted old animals elicits a pattern rather similar to that observed in cultures derived from the young ones. Some problems in interpreting data might arise from the fact that cells from older animals do contain larger amount of proteins: it may be argued that the observed age-related decrease could be due to the fact that [³H]TdR incorporation is corrected relating to the protein content. However, when the comparison is performed using dpm values directly without correcting for protein content, data from the three animal groups show patterns similar to those reported in Figure 4.

These findings add information on the corrective action of the gland on nonimmunologic functions that we have been studying for some time (20). We observed restoring influences on the adrenergic system and on the β₁, in particular (21), but it is worth noting that the thymus has fairly

Table 3. Age-Related Modifications and Thymus-Induced Recovery of Hepatocyte DNA and Protein Content

<table>
<thead>
<tr>
<th>Animal Models</th>
<th>n</th>
<th>Protein (µg/well ± SEM)</th>
<th>DNA (µg/well ± SEM)</th>
<th>DNA/protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>8</td>
<td>91 ± 10</td>
<td>1.58 ± 0.15</td>
<td><strong>.017</strong></td>
</tr>
<tr>
<td>Old</td>
<td>8</td>
<td>146 ± 17</td>
<td>2.93 ± 0.25</td>
<td><strong>.020</strong></td>
</tr>
<tr>
<td>Old + thymus</td>
<td>7</td>
<td>121 ± 16</td>
<td>1.78 ± 0.21</td>
<td><strong>.015</strong></td>
</tr>
</tbody>
</table>

Notes: DNA and protein contents for each animal have been calculated as the mean of data obtained from time 0 to 54 h (150,000 viable cells per well); values reported in the table refer to mean of values from all mice studied. Data were evaluated using analysis of variance, followed where appropriate by Student-Newman-Keuls’ test (significance level: p < .05). * Is put between significantly different pairs of values.
widespread actions also involving modulation of hormone serum levels (3,22) and some morphometric parameters of hepatocytes (1). In this regard the comparison of the present results with previous data showing a corrective action of the thymus on hepatocyte nuclear volume and ploidy degree, both found to be increased in old mice, is particularly interesting (2,23). In fact, data presented here show an increase in the values for total DNA content in hepatocytes from old animals, values found to be decreased significantly in hepatocytes from thymus-grafted old mice. Because endocrine balance influences RNA and DNA synthesis (24,25), its modulation could mediate the corrective action of the thymus observed on both the rate of DNA synthesis and DNA content. In this context, the intriguing hypothesis of an unblocking action on the division process of polyplloid liver cells cannot be excluded.

The considerations made above give rise to a picture of interacting mechanisms and factors that are far from clear as numerous hormonal and growth factors are involved in the initiation of liver cell proliferation (26). Certainly, the thymus acts on targets of paramount importance for hepatocyte physiology such as stimulated and unstimulated DNA synthesis and cell division. Thus, it will be interesting to investigate both the mechanisms of the thymic actions as well as the possibility that some thymic factors mimic such actions. In fact, recent findings showed the influence of a thymic crude aqueous extract on the rate of in vivo DNA synthesis and on adrenocortical density in mice (27).

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REFERENCES


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