Epidermal growth factor stimulated protein kinase shows similar activity in liver of senescent and adult mice

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It was recently reported [(1983) Nature 306, 617–620] that tyrosine protein kinase activity associated with EGF receptor was absent from senescent human cultured fibroblasts, which are known to have the same number of receptors as young human cultured fibroblasts. We have measured in both adult and senescent C57 black mice the number of EGF receptors, the activity of their associated tyrosine kinase and the activity of the protein phosphatase which dephosphorylates the EGF receptor. We found our results in both groups of animals to be similar which indicate that the observations made in cultured fibroblasts cannot be generalized to all mammalian tissues.

Epidermal growth factor *Receptor* *Tyrosine kinase* *Senescence* *Liver*

1. INTRODUCTION

Hormones exert their effects by way of receptors on the surface or in the cytoplasm of target cells [1]. Likewise there exists a sizable body of evidence that tissue responsiveness to hormones declines with age [2,3]. This decline may be caused by changes in receptors or in the sequence of enzymatic reactions provoked by the interaction of the hormone with its receptor. There may be alterations in receptor number, binding affinity or enzymatic activity associated with the receptor which is the first step of the hormonal effect at the cellular level. Growth factors are in many respects similar to hormones and exert their effects through binding to a surface receptor. Epidermal growth factor (EGF) binds to a membrane protein of 170 kDa possessing a tyrosine protein kinase activity stimulated by EGF [4]. A recent publication [5] indicates that in fibroblasts (a model generally admitted to reflect the changes observed in senescent animals) after a great number of passages if the number of EGF receptor is unchanged, the tyrosine protein kinase activity associated with this receptor has on the contrary disappeared from these cells.

These results were also the first to indicate a dissociation between receptor and kinase activity at the same receptor molecule. Concerning this last point, recent observations have shown that in some insulin-resistant diabetics, if the insulin receptor capacity is unchanged, the tyrosine protein kinase activity associated with it is strongly diminished [6].

These results prompted us to measure the number of EGF receptors and associated tyrosine protein kinase activity in the liver of adult and senescent mice. In this tissue these activities are well characterized [7,8] and as in A 431 cells [4] and fibroblasts [9] the EGF receptor undergoes autophosphorylation on tyrosine residues after EGF binding. As a tyrosine protein phosphatase modulates the phosphorylation induced by EGF we have also measured this activity in membrane liver of both groups of animals.

2. MATERIALS AND METHODS

2.1. *Animals*

C57 black male mice of different ages were provided by the CNRS animal house at Villejuif and were fed with Purina chow.

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2.2. *Microsomal fraction preparation*

Mice had access to food and water ad libitum. They were killed by decapitation. Liver was homogenized in 3 vols of 50 mM Tris, pH 7.6, 0.25 M saccharose containing 1 mM PMSF and 5 mM iodoacetamide. After elimination of nuclei and mitochondria by centrifugation for 15 min at 12000 × *g*, microsomes were obtained by centrifugation of the supernatant for 1 h at 100000 × *g*. They were washed once with 5 mM Tris, pH 7.5, containing 1 mM PMSF and 5 mM iodoacetamide [7]. This fraction of microsomes and sinusoidal membranes containing the EGF receptor were stored at −80°C until use.

2.3. *EGF receptor determination*

The assay, outlined by Nexo et al. [10], is as follows: microsomal fraction (about 10 mg protein per ml in 5 mM Tris-HCl, pH 7.5) was diluted with 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% (w/v) bovine serum albumin to a final concentration of 0.5 μg protein per ml.

An aliquot of the diluted membrane suspension (100 μl) was added to a mixture composed of 100 μl ^{125}I-EGF (about 70000 cpm) and 100 μl standard solution of unlabeled EGF in phosphate buffer. After 60 min at room temperature, the membranes were collected and washed on Millipore EGWP filters (0.2 μm pore size) for measurement of bound radioactivity. Non-specific binding, determined by using a 1000-fold excess of unlabeled EGF, was subtracted to obtain specific ^{125}I-EGF binding.

2.4. *Membrane phosphorylation*

The assay was performed in 35 μl of reaction mixture containing the following: microsomal fraction, 30 μg protein; 50 mM Tris-HCl buffer (pH 7.6); 2 mM $MnCl_2$; 2×10^{-7} M EGF; 5 mM *p*-nitrophenyl phosphate; 5 μg serum albumin; 1 μM (0.5 μCi) [γ-^{32}P]ATP.

The reaction tubes were preincubated at 33°C for 6 min. The reaction was initiated by addition of labeled ATP and incubation was continued for 4 min at 33°C. The reaction was stopped by the addition of 3-fold concentrated sample buffer and the proteins were subjected to electrophoresis in 15% acrylamide-0.1% bisacrylamide slab gels using the Laemmli [11] discontinuous system. After staining with Coomassie blue and drying, autoradiography was performed by enclosing a flashed Kodak royal X-OMAT A R film between the gel and a Dupont lighting screen at −70°C for 1–2 days [12].

Phosphotyrosine protein phosphatase was measured by dephosphorylation of *p*-nitrophenol phosphate (PNPP) according to Shriner and Brautigan [13]. Briefly membranes diluted in 50 mM Hepes (pH 7.0), 0.5 M EDTA, 25 mM 2-mercaptoethanol and 0.5 mg/ml serum albumin were incubated with 0.01 M PNPP for 5 min at 30°C. After addition of 0.1 M sodium carbonate the absorbance at 410 nm was recorded. Protein determination was done according to Bradford [14]. EGF was prepared according to Savage and Cohen [15]. Chemicals were high purity grade (Sigma, Merck). [γ-^{32}P]ATP (50000 Ci/mmol) and ^{125}I-EGF (100 μCi/μg) were from Amersham.

3. RESULTS AND DISCUSSION

The binding of EGF to microsomal fractions of liver of young and senescent mice is shown in table 1. It can be seen that this binding is comparable in both groups. Assuming that a C57 black mouse liver corresponds to 10^9 cells and 50 mg proteins, these values correspond to a receptor number per cell of 30000. These values are comparable to those of Moriarity and Savage [16] and Earp and O'Keefe [17] using isolated hepatocytes. This number may be underestimated as some receptors may be masked and detected only by antireceptor antibodies [18].

Table 1

Summary of binding data from liver membrane fraction in adult and senescent mice

Expt	8 weeks	24 months
1	327 ± 28	361 ± 20
2	299 ± 37	331 ± 32
3	618 ± 44	698 ± 28
4	239 ± 27	214 ± 28
5	314 ± 27	282 ± 17
Mean	359.4 ± 32.6	377.2 ± 25

Mean ± SE of 4 separate animals: binding activity values are expressed as pg EGF bound per 50 ng microsomal protein. Conditions were as described in section 2

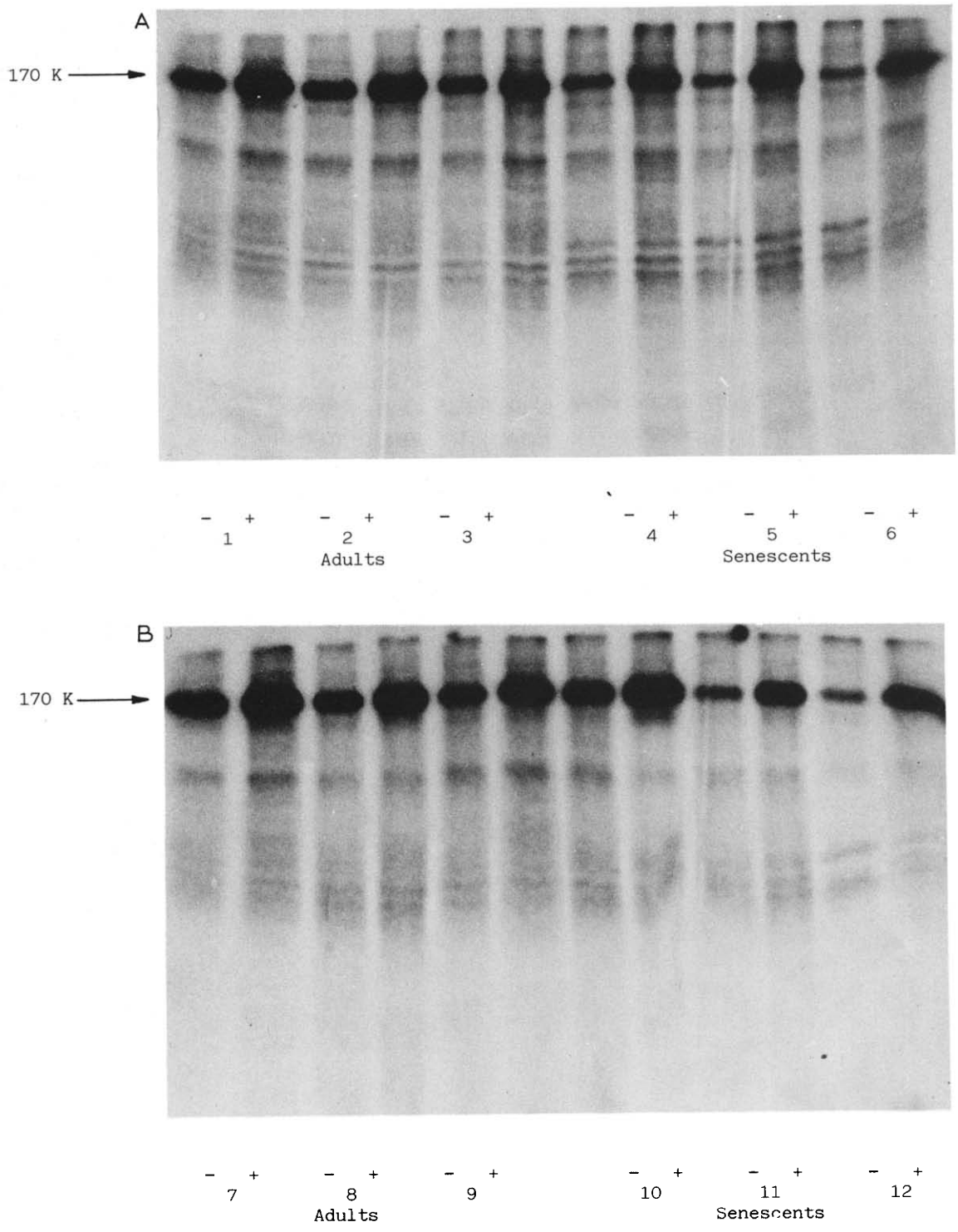

Fig.1. SDS-polyacrylamide gel electrophoresis of mouse liver microsomes followed by autoradiography (acrylamide concentration 15%). (−) No EGF; (+) EGF added; adults, 8 weeks old; senescents, 24 months old. (A) 1–3, adults; 4–6, senescent mice. (B) 7–9, adults; 10–12, senescent mice.

The autophosphorylation of the microsomal membrane fraction in the absence and presence of EGF is shown in fig.1 (autoradiogram) and table 1. In the presence of EGF one can see a strong augmentation of the phosphorylation of a band of 170 kDa (arrow) which corresponds to EGF receptor [4,7]. The patterns and intensity of phosphorylation are the same in both groups (fig.1).

In table 2 are indicated the kinase activity obtained after cutting out and counting the 170 kDa band. Both the kinase activity and the degree of stimulation by EGF are similar in the 2 groups.

Phosphorylation in the absence of EGF may be due to adsorbed EGF from blood or phosphorylation by another protein kinase present in microsomal fractions.

Similar results have been obtained in C3H mice aged 10 weeks and 20 months, respectively, and in Wistar rats aged 3 months and 20 months (not shown).

Our results showing no difference between the EGF-stimulated protein kinase activity of liver of either adult or senescent animals differ from those of Carlin et al. [5]. These authors reported that senescent human fibroblasts [19] lost their capacity to growth in response to EGF (a result also found by Tsuji et al. [20]). Although the receptor number was the same in young and senescent human fibroblasts, the EGF-stimulated protein kinase activity dropped in senescent fibroblasts. This system is of course very different from the mouse liver system although it is generally believed to reflect the change occurring in aging animals. We have measured the EGF binding and protein kinase activities in a fraction enriched in membranes whereas Carlin et al. immunoprecipitated the EGF receptor from whole cells. They may have coprecipitated phosphatases or proteases which could change according to the age of fibroblasts. In mouse liver membrane the tyrosine protein phosphatase activities are similar in adult and senescent animals as shown in table 3. Other unknown factors may also inhibit the EGF-dependent protein kinase activity.

One may object that aging differs from tissue to tissue and that liver does not age as do fibroblasts, but that murine liver also ages as shown by the poor quality of compensative hypertrophy after hepatectomy in senescent rats [21]. A decrease in

Table 2

Stimulation of phosphorylation of 170 kDa band by EGF

8 weeks (cpm)	nm ^{32}P per min	Stimulation by EGF	24 months (cpm)	nM ^{32}P per min	Stimulation by EGF
1 – 780	3.9	2.10	4 – 756	3.78	1.78
+1643	8.21		+1343	6.71	
2 – 768	3.84	2.10	5 – 670	3.35	2.39
+1613	8.06		+1605	8.02	
3 – 664	3.32	2.57	6 – 467	2.33	2.74
+1708	8.54		+1282	6.41	
7 –1146	5.73	2.09	10 –1232	6.16	2.22
+2391	11.95		+2734	13.67	
8 – 922	4.61	2.12	11 – 528	2.64	2.28
+1955	9.77		+1205	6.02	
9 – 861	4.3	1.81	12 – 318	1.59	3.35
+1561	7.8		+1066	5.33	
Mean		2.13			2.45

cpm and activity in ng ^{32}P per min of the 170 kDa band of fig.1. The 170 kDa band was cut from the dried gels and counted by the Cerenkov effect. –, no EGF; +, EGF added

Table 3
p-Nitrophenol phosphatase activity in liver membrane of adult and senescent mice

8 weeks		24 months	
A_1	45.3	S_1	55.6
A_2	36.1	S_2	48.8
A_3	56.1	S_3	52.3
A_4	31.7	S_4	52.3
Mean	42.30		52.25

PNP phosphatase activity was measured as indicated in section 2. Membrane concentration was 1 mg/ml, results are expressed in nM *p*-nitrophenol per min

the number of various hormonal receptors has also been described in the liver of senescent animals [3].

In conclusion our results show that in mouse liver there is no difference in the adult and senescent animal concerning the first step of EGF action at the membrane. However, there are no data on the further steps of EGF action in senescent animals. More generally it would be interesting to examine thoroughly the response of senescent animals to normal and tumoral growth factors.

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