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Effect of 2-Acetylaminofluorene on the Binding of Epidermal Growth Factor to Microsomal and Golgi Fractions of Rat Liver Cells[1]

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ABSTRACT

The livers of rats fed the hepatocarcinogen 2-acetylaminofluorene (0.02%) with chow showed a sharp decrease in the binding of epidermal growth factor to microsomes and Golgi fractions. The binding to the latter decreased from 15.3% specific binding per 0.1 mg protein in controls to 9.4% after 2 days and reached a nadir of 0.8% after 21 days. The binding to microsomes decreased from 26.3% specific binding per 0.5 mg protein in the controls to 17.4% after 4 days and reached a nadir of 7.5% after 46 days. The low binding which persisted until the end of the experiment (85 days) was due to the apparent decrease in the number of receptors without significant changes in their affinity. Also, there was only partial recovery in rats fed 2-acetylaminofluorene for 90 to 107 days and taken off the carcinogen for 30 to 75 days. *In vitro,* neither 2-acetylaminofluorene nor its metabolites hydroxy- and acetoxy-2-acetylaminofluorene significantly decreased epidermal growth factor binding to the isolated microsomal fraction.

INTRODUCTION

The receptors to EGF[3] bind, in addition to EGF itself, some TGF, type I, produced by normal embryonic and some transformed cells (for reviews, see Refs. 6, 8, 9, 17, and 24). A decrease in the EGF binding to its receptors has been found in partially resected regenerating liver (14), in cells treated with phorbol esters (19), in certain experimental cancers (10–12, 15, 18), and in human hepatoma (7). Although alterations of the EGF binding to its receptors in the course of chemical hepatocarcinogenesis have not been studied systematically, such studies could be of interest since EGF is not only an almost ubiquitous growth factor but also one of the most potent stimulants of hepatocyte DNA synthesis. The potential importance of such a study for oncology has been stressed recently by the finding of extensive homology of the rat TGF, type I, to EGF (20) and homology of certain domains of the EGF receptor in humans to v-*erb*-B oncogene proteins (13).

We have designed experiments to determine whether the hepatocarcinogen 2-AAF causes changes in the EGF binding to rat liver microsomal and Golgi fractions. The results reported here show that rats fed 2-AAF demonstrated an early and sustained decrease in the binding of EGF to these fractions, and these effects could not be reproduced with 2-AAF or its metabolites added to these fractions *in vitro.*

MATERIALS AND METHODS

Adult male Fischer rats (180 to 200 g) were fed a basal diet containing 0.02% 2-AAF. The rats were sacrificed by exsanguination under ether anesthesia after 2, 4, 8, 21, 34, 46, and 85 days. Control rats received regular laboratory chow. Each group consisted of 4 to 9 animals, and all the determinations were done in quadruplicates.

The livers were perfused *in situ* with cold 0.9% NaCl solution (saline), excised, and homogenized in 0.25 M sucrose. This and all subsequent procedures were conducted at 4°. Liver homogenates were centrifuged at 10,000 × *g* for 10 min, and the supernatant was removed and subjected to centrifugation at 105,000 × *g* for 75 min. The pellet was homogenized with buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-2 mM EDTA-10 mM $MgCl_2$-10 mM $CaCl_2$-50 mM NaCl-5 mM KCl, pH 7.4). The homogenate contained a crude microsomal fraction. Part of it was kept at −70°. The remaining aliquot was resuspended in 1.15 M sucrose; carefully inserted beneath a discontinuous gradient of 0.25, 0.60, and 0.86 M sucrose; and centrifuged in a Beckman ultracentrifuge (Sw-28 rotor) at 83,000 × *g* for 165 min. Three Golgi fractions were obtained, but the amount of the light fraction was not sufficient for binding studies. The verification of the Golgi nature of the fraction was done with electron microscopy and by the determination of the enzyme marker galactosyltransferase (4). In 24 separate experiments performed on both control rats and rats fed 2-AAF for 2 to 85 days, no differences were found in the EGF binding to the Golgi intermediate and heavy fractions; therefore, the fractions were combined. The Golgi fraction was diluted with water, pelleted by centrifugation at 105,000 × *g* for 90 min, resuspended in the buffer, and kept at −70°.

For the receptor binding assays, ^{125}I-labeled EGF was prepared by the chloramine-T method to a specific activity of 80 to 100 mCi/mg. The membranes were diluted with buffer (composition indicated above) to a concentration of protein of 5 mg/ml for the microsomes and 1 mg/ml for the Golgi fraction. The incubation mixture contained 0.3 ml of the buffer with bovine serum albumin added (2 mg/ml), 0.1 ml of a membrane suspension, and 0.1 ml of the labeled ligand (40,000 cpm in buffer). Incubation was carried out at 4° for 48 hr. In preliminary experiments, the incubation was conducted at 37, 25, 16, and 4° and for various lengths of time. The most consistent results were obtained, and the EGF binding reached a steady state after 40 to 48 hr at 4°.

After the incubation, aliquots of 0.2 ml were removed and added to 0.8 ml of cold buffer. Bound and free ligands were separated by centrifugation in the Beckman microfuge (Model B) for 5 min. The pellet was washed with 1 ml of cold buffer and recentrifuged. The radioactivity was measured in a Beckman gamma counter (Model 300). The results were expressed as the percentage of specific binding per 0.5 mg of protein for microsomes and per 0.1 mg of protein for Golgi membranes. Statistical significance of the differences was assessed by the parametric *t* test.

In vitro studies were performed by the addition to the incubation mixture of 2-AAF, hydroxy-AAF, or acetoxy-AAF (dissolved in DMSO) to a final concentration of 25, 50, 100, and 200 μg/ml with 5% DMSO as control.

Affinity studies were performed with the concentrations of the cold ligand of 0, 0.5, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, 100, and 200 ng/ml, the highest concentration being used for the determination of the nonspecific binding.

EGF was purchased from Collaborative Research, Inc., Lexington,

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[3] The abbreviations used are: EGF, epidermal growth factor; 2-AAF, 2-acetylaminofluorene; DMSO, dimethyl sulfoxide; TGF, transforming growth factor(s); hydroxy-AAF, hydroxy-2-acetylaminofluorene; acetoxy-AAF, acetoxy-2-acetylaminofluorene.

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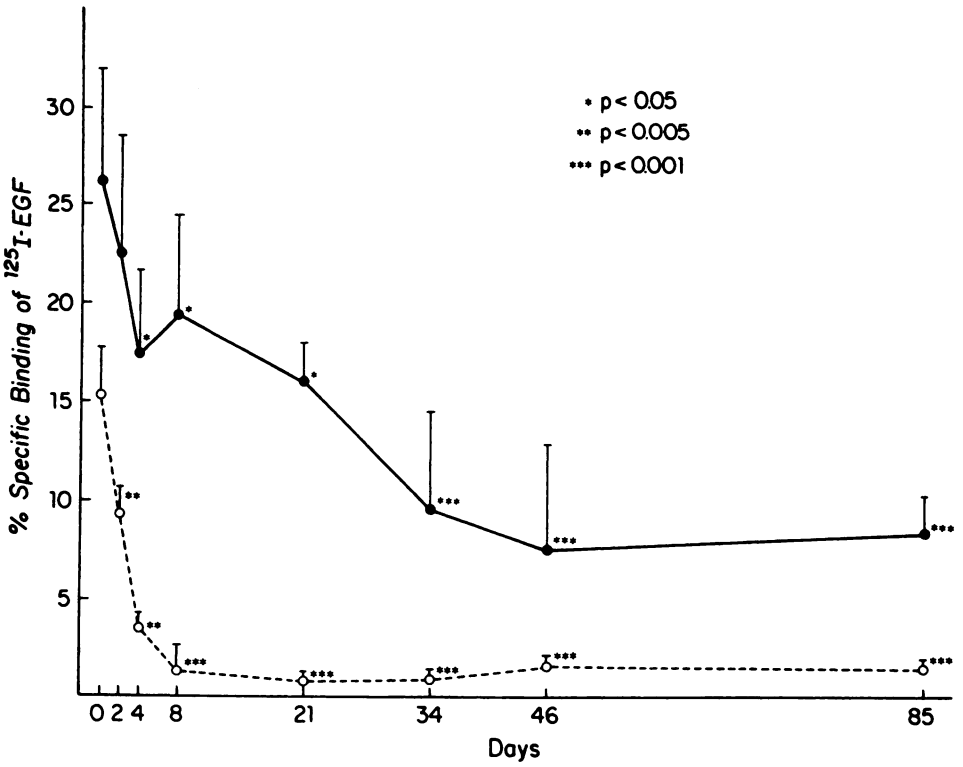

Chart 1. Binding of ^{125}I-labeled EGF to microsomal (●) and Golgi (○) fractions of the rat liver cells. The animals were fed 2-AAF for 2 to 85 days. Day 0 shows the binding in the control group. Probability values are given for the differences from the control group. *Bars*, S.D.

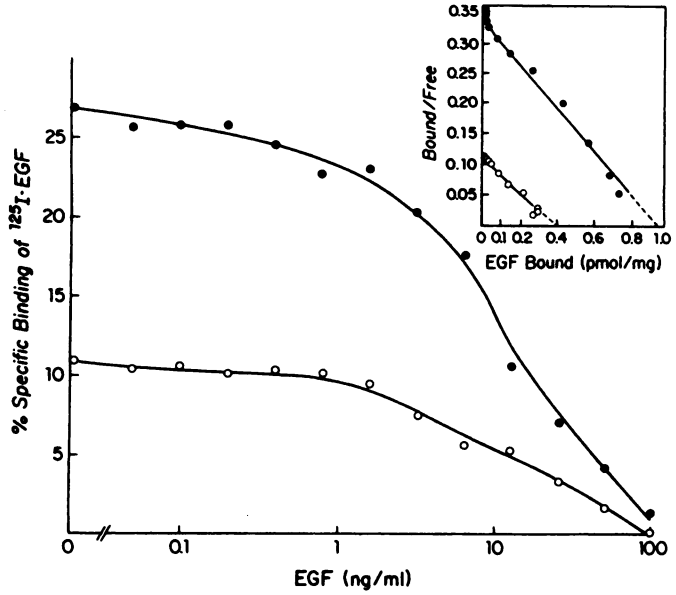

Chart 2. Competition curve and Scatchard plot (*inset*) of the EGF binding (per 0.5 mg protein) to the microsomes of the liver cells of the control rats (●) and rats fed 2-AAF for 46 days (○). At 46 days, the binding by the microsomes reached nadir.

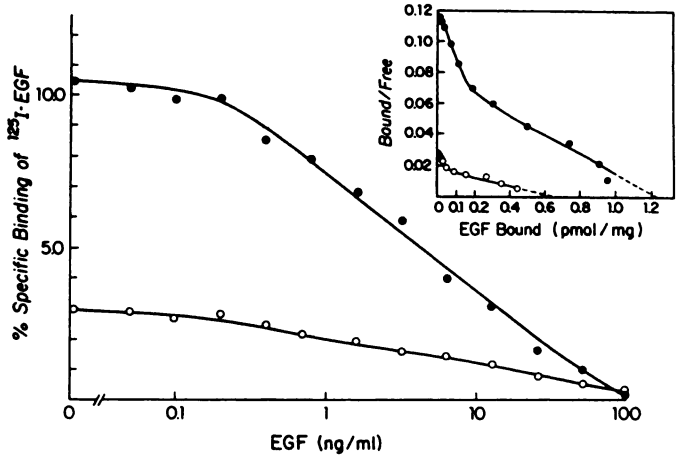

Chart 3. Competition curve and Scatchard plot (*inset*) of the EGF binding (per 0.1 mg protein) to the Golgi fraction of the liver cells of control rats (●) and rats fed 2-AAF for 21 days (○). At 21 days, the binding reached its nadir.

MA; 2-AAF was from Aldrich Chemical Co., and the rat diet was prepared by Dyets, Inc., Bethlehem, PA. Rats were purchased from Simonsen Laboratories (Gilroy, CA). Hydroxy-AAF and acetoxy-AAF were the kind gift of Dr. J. Miller, McArdle Laboratory, Madison, WI.

RESULTS

Chart 1 shows an early, sharp decrease in the EGF binding to the liver fractions of rats fed 2-AAF. A decrease in the EGF binding to the Golgi fraction was statistically significant as early as 2 days after the beginning of the 2-AAF feeding: from 15.3 ± 2.6% in controls to 9.4 ± 1.2% in rats fed 2-AAF ($p < 0.005$). After 4 days, the binding decreased further to 3.5 ± 0.7%, by 8 days to 1.4 ± 1.2% ($p < 0.001$), reached a nadir of 0.8 ± 0.6% by 21 days ($p < 0.001$), and remained low thereafter to the end of the experiment.

A less marked decrease occurred in the EGF binding to the microsomal fraction (Chart 1). It reached statistical significance after 4 days, decreasing from 26.3 ± 5.8% to 17.4 ± 4.4% ($p = 0.013$). A nadir of 7.5 ± 5.5% was reached after 46 days ($p < 0.001$).

Charts 2 and 3 show the competition curves for microsomal and Golgi fractions for the control rats and rats fed 2-AAF (the latter, at the nadir of the EGF binding). The Scatchard plot of the EGF binding to microsomes presented a straight line. The binding

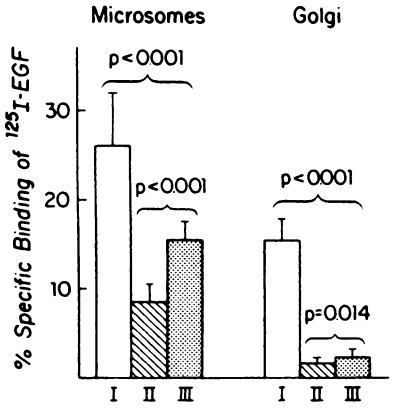

Chart 4. Binding of EGF to microsomal and Golgi fractions in the liver cells of the control rats (*I*), rats continuously fed 2-AAF for 85 days (*II*), and rats fed 2-AAF for 90 to 107 days and taken off the carcinogen for 30 to 75 days (*III*). *Columns*, mean of 4 to 9 animals; *bars*, S.D.

capacity was 0.96 pmol/mg in the control rats and 0.40 pmol/mg in the rats fed 2-AAF. The affinity of the receptors was the same in both groups (1.45 nM).

The Scatchard plot of the EGF binding to the Golgi fraction was curvilinear with the following characteristics. The high-affinity receptors showed K_1 0.40 nM in both control and 2-AAF-fed rats; the binding capacity was 0.43 pmol/mg in control rats, and 0.13 pmol/mg in rats fed 2-AAF; the low-affinity component showed K_2 1.58 nM in control rats and 3.42 nM in rats fed 2-AAF, with the corresponding binding capacity of 1.25 and 0.65 pmol/mg.

In another series of experiments, 2-AAF was given for a period of 90 to 107 days, after which it was removed from the diet for an additional period of 30 to 75 days. Chart 4 shows that, in both microsomal and Golgi fractions, the binding of EGF remained sharply reduced in comparison to the control rats and was only slightly higher than in the rats sacrificed after 85 days of 2-AAF feeding. In control rats, in rats fed 2-AAF for 85 days, and in the "on-off" group, the EGF binding to the microsomes was 26.3 ± 5.8%, 8.4 ± 2.1%, and 15.5 ± 1.9% per 0.5 mg of protein, and to the Golgi fraction, it was 15.3 ± 2.6%, 1.6 ± 0.4%, and 2.6 ± 0.7% per 0.1 mg of protein.

In vitro experiments with the EGF binding to normal liver microsomes incubated with 2-AAF, hydroxy-AAF, and acetoxy-AAF showed that, in the presence of 5% DMSO alone, the binding was 85% of the control. 2-AAF and its metabolites in the concentrations of 25 to 100 μg/ml had no effect on the binding, whereas in the concentration of 200 μg/ml, they decreased the binding to 76.5% of the control.

DISCUSSION

In the described experiments (repeated 3 times), the hepatocarcinogen 2-AAF caused an early, sharp, and sustained decrease in the EGF binding to microsomal and Golgi fractions in rats. Only one class of receptors was found in the microsomes, and they exhibited a sharp decrease in their number without changes in their affinity. The Golgi fraction showed, on the other hand, the presence of 2 classes of receptors. In rats fed 2-AAF, the number of high-affinity receptors decreased, but their affinity did not change. The low-affinity receptors demonstrated a decrease in their number and affinity.

Endoplasmic reticulum (the main component of the microsomes) is the putative site of the synthesis of proreceptors with the final glycosylation and processing taking place in the Golgi system (25). We could find no references concerning the differences in the binding of EGF by different intracellular receptors, but it was reported recently in relation to insulin that the affinity of the Golgi receptors was much higher than that of plasma membranes (21). It is possible that the higher affinity of the Golgi receptors for EGF found in our experiments can be explained by the higher purity of the fraction, but the latter cannot possibly explain the presence of 2 classes of receptors in the Golgi fraction in comparison to one class in the microsomes. In any case, in further experiments with hepatocarcinogens, the possibility of differential changes in different intracellular receptors should be taken into account.

The total or partial loss of the EGF receptors was reported in chemically or spontaneously transformed liver cells, in W8 cells transformed by 2-AAF (15), and in human hepatoma (7). In rat hepatomas, both EGF binding to microsomes and EGF-induced receptor autophosphorylation were diminished or absent (3). A significant decrease in the affinity of EGF receptors is caused by phorbol esters (15, 19).

The changes found in the EGF binding to subcellular fractions are among the earliest reported. Other known early changes include the induction of resistance to cytotoxins observed after 24 hr (5), increased activity of hepatic acid DNase and β-glucuronidase seen after 3 days (2), and electron-microscopic changes in endoplasmic reticulum and the Golgi system described after 1 week (16). Numerous biochemical changes (22) appear much later; no hyperplasia appears histologically before 4 weeks, and no nodules appear before 6 weeks.

Several possible explanations of the decreased EGF binding in the course of hepatocarcinogenesis can be offered. One possibility is a true decrease in the number of receptors. This seems likely in view of the similar finding in several experimental hepatomas (3). Another possibility is the production by the cells of TGF, type I (23), competing with EGF for its receptors (20). In our experiments, acid-ethanol extracts of the livers of rats fed 2-AAF did not possess any activity competing with EGF; therefore, this possibility seems unlikely. An equally unlikely explanation is the production of TGF, type II, since the latter does not decrease and, on the contrary, increases the number of EGF receptors and suppresses their down-regulation (1). Finally, an intriguing possibility is the dissociation of the ligand-binding and tyrosine-phosphorylating domains of the receptors: peptides derived from the cytoplasmic domain of the EGF receptor exhibited homology to certain oncogene protein sequences (13). This possibility, though, seems less likely in view of the reported decrease in the receptor autophosphorylation in experimental hepatomas (3).

Receptors to EGF share with the receptors to insulin, somatomedin-C, and platelet-derived growth factor a common property: tyrosine phosphokinase. Our studies[4] showed that the changes in the insulin binding were less pronounced than, but highly positively correlated with, the changes in the EGF binding. Therefore, it seems probable that the changes reported here are not specific for EGF receptors only.

Several observations point to possible intimate relations among EGF, EGF receptors, and carcinogenesis, *e.g.*, a correlation between the tumorigenicity and a decrease in EGF binding in several rat liver cell lines (15), extensive homology of TGF,

[4] A. Lev-Ran, Z. Josefsberg, B. I. Carr, G. Barseghian, D. Hwang, J. Nat. Cancer Inst., in press.

type I, to EGF (20), and a homology of certain avian oncogene proteins to some proteins derived from the EGF receptor (13). Our observations of the early appearance of the changes in EGF binding in the course of chemical hepatocarcinogenesis point to the same direction and deserve further study.

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