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Changes in the Binding Capacity of Hepatic Membranes for Epidermal Growth Factor during Multistage Hepatocarcinogenesis in Rats

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To study changes in hepatic capacity for binding epidermal growth factor (EGF) during 2-acetylaminofluorene (2-AAF)-induced, multistage hepatocarcinogenesis, a 5 cycle protocol of discontinuous 2-AAF administration was used to produce hepatocarcinogenesis in rats. The hallmark of the 5 cycle protocol is that rats fed 1 to 3 cycles of 2-AAF are at low risk for cancer, while rats fed 2-AAF for 4 or 5 cycles are at high risk for cancer. EGF binding by liver membranes was found to be lowered to 20–25% of control throughout the 5 cycle regimen. When the persistence of lowered EGF binding was tested by returning rats fed 2-AAF for 1 to 3 cycles to diet without 2-AAF for 3 weeks, binding was found to recover to 80 to 90% of values for control rats. In contrast, for rats fed 2-AAF for 4 or 5 cycles, EGF binding capacity remained low, 30 to 40% of control, following placement of rats on diet without 2-AAF for 3 weeks. Immunochemical analysis indicated a close correspondence between changes in EGF receptor levels and changes in the above EGF binding levels. These studies show that during the 2-AAF protocol, the 2-AAF-mediated loss in hepatic EGF binding capacity and EGF receptor protein undergo a transition from a reversible loss to a persistent loss in binding capacity, and EGF receptor protein, as rats underwent a change from low to high risk for developing hepatocarcinomas. The persistent decrease in hepatic EGF binding level may be associated with the progression stage of hepatocarcinogenesis.

Carcinogenesis is a multistage process involving alterations in gene expression and cell growth control. One of the hallmarks of this process is a change in cell response to growth regulation by hormones (1). Deviations from the normal regulatory process, such as abnormal levels of hormone ligand, modulation of the number and affinity of hormone receptors, mutations in the hormone receptor, and alterations in post-receptor signal transduction pathways, have all been implicated in the process of abnormal growth control leading to the development of altered precancerous cell populations and subsequent progression to malignant tumor formation (2-6).

Epidermal growth factor (EGF) is a potent mitogen known to be present in liver tissue and to be able to stimulate hepatocytes in cell culture to proliferate. Furthermore, administration of the hepatocarcinogens, 2-acetylaminofluorene (2-AAF) and diethylnitrosamine (DEN), to rats has been reported to result in a decrease in the membrane binding of EGF (7-9). The ability to disrupt this normal growth regulatory pathway may be an important property of carcinogens and a critical factor in the multistage process of cancer development.

In this study, we have employed an experimental carcinogenesis model to assess changes in EGF binding during multistage cancer development. This cancer model uses discontinuous, 5-cycle, feeding of 2-AAF over 19 weeks, where each cycle consists of 3 weeks of 0.05% 2-AAF administration followed by 1 week of feeding a control basal diet without 2-AAF (10). As previously reported, rats on this protocol showed a low risk for cancer if administered 2-

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AAF for only 3 cycles, and a high rate cancer development if administered 4 or more cycles of 2-AAF (11, 12). Thus, use of this protocol furnished an opportunity to assess possible changes in EGF function at a critical stage in liver carcinogenesis. The findings from this study demonstrated that 2-AAF-mediated a decrease in EGF binding, and EGF receptor protein, that was reversible through 3 cycles of 2-AAF exposure, but became irreversible following a 4th cycle of 2-AAF administration.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. 2-AAF was purchased from the Aldrich, Chemical Co. (Milwaukee, WI). Mouse [^{125}I]-EGF and EGF were obtained from the NEN Research Products, DuPont (Boston, MA).

Animals and treatment. Male Sprague-Dawley rats (180-200 g) were purchased from Sasco Inc. (Omaha, NE). They were maintained on a 12-h light and 12-h dark daily cycle, given food and water *ad libitum* and acclimated to their environment at least 1 week prior to their use in experiments. To induce cancer, groups of rats were maintained on the semi-synthetic basal diet (13) containing 0.05% 2-AAF according to a protocol for inducing hepatocarcinogenesis (10). This protocol uses a discontinuous, 5-cycle feeding of 2-AAF over 19 weeks with each cycle consisting of 3 weeks of 0.05% 2-AAF followed by 1 week of feeding the basal diet without 2-AAF. Age- and sex-matched control rats were fed the semisynthetic basal diet continuously during the period that experimental rats were in cyclic feeding regimens. Rats maintained beyond a particular period of 2-AAF administration were fed the semisynthetic basal diet.

Preparation of membrane fractions. Rat livers were excised and homogenized in 0.25 M sucrose solution and microsomal membranes prepared using a modification of a method described by Josefsberg, Z. *et al.* (9). Briefly, homogenates were filtered through cheese cloth and centrifuged at 10,000 $\times$ g for 10 min at 4°C. The resulting supernatant was recentrifuged at 105,000 $\times$ g for 75 min to give a microsomal pellet. Microsomes were resuspended by hand-homogenizing in HEPES+ Buffer (50mM HEPES (pH 7.4), 2mM EDTA, 10mM $MgCl_2$, 10mM $CaCl_2$, 50mM NaCl, and 5mM KCl) to a final concentration of 5 mg/ml and stored at -70°C until used in binding assays.

Plasma membrane enriched fractions were prepared as previously described (14). Frozen liver tissue (1.5g) was crushed in liquid nitrogen and resuspended in a hypotonic buffer containing 10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 20μg/ml phenylmethylsulfonyl fluoride (PMSF), and 500μg/ml of the following protease inhibitors: Pepstatin A, Leupeptin, and Aprotinin. The tissue was then homogenized with 6-10 strokes in a Dounce glass homogenizer in 5-8 ml of cold hypotonic buffer. This homogenate was centrifuged at 300 $\times$ g for 10 min, and the resulting pellet was resuspended and repelleted by centrifugation. The corresponding 300 $\times$ g supernatants were pooled and centrifuged at 4000 $\times$ g for 10 min and the pellet resuspended and repelleted as before. The 4000 $\times$ g supernatants were combined and layered over a 5 ml cushion of the hypotonic buffer containing 0.25M sucrose, and centrifuged for 1 hr at 100,000 $\times$ g. The membrane layer was extracted, centrifuged and pelleted. The membrane pellet was homogenized in phosphate-buffered saline containing 20μg/ml of PMSF, and stored at -70°C until used in Western immunochemical experiments. Differences in membrane recovery were normalized based on immunochemical measurements of membrane ATPase (Anti-Rat Na, K ATPase alpha 1 fusion protein, Upstate Biotechnology Incorporated, Lake Placid, NY) at the time of EGF receptor determination. Protein concentrations were measured using a modified Lowery method employing bovine serum albumin as a standard (15).

Hormonal binding assay. Microsomal preparations were diluted with HEPES+ Buffer to a protein concentration of 5 mg/ml. A reaction mixture contained 0.3 ml of HEPES+ buffer, BSA (2 mg/ml), 0.1 ml of membrane suspension, and 0.1 ml of [^{125}I]-EGF (40,000 dpm) was added to a 1.5 ml microtube. The tubes were incubated for 40 hrs at 4°C with constant shaking, and then centrifuged at 8800 $\times$ g for 10 min to pellet the membranes (9). The supernatant was aspirated, pellets washed by resuspension in 1 ml of cold HEPES+ buffer, membranes re-pelleted by centrifugation and the wash removed by aspiration. The ends of the microtubes containing the microsomal pellets were cut off, and the radioactive content was measured by gamma counting. Percent specific binding was determined by subtracting nonspecific binding from total binding, and results were expressed as percent specific binding/0.5 mg protein.

Immunochemical analysis of EGF receptor. Aliquots of enriched plasma membrane (30-50 μg) were dissolved in sample buffer comprised of 0.125 M Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol (16). Samples were electrophoresed on a 4-15% Tris-HCl gel (Bio Rad, Hercules, CA) and electroblotted to a polyvinilidene (PVDF) membrane from Micron Separations, Inc. (Westborough, MA). The conditions used were 126 V using constant voltage for 55 min in 0.025 M Tris-HCl/ 0.192 M glycine buffer, (pH 8.3). Blots were probed with monoclonal antibody to human EGF receptor (ATCC Clone L-4451, Calbiochem, La Jolla, CA). Lanes containing membranes from A431 cells (ATTC CRL 1555, ATCC, Rockville, MA) were used as a positive control the presence of EGF receptors. ECL Western Blotting procedures from Amersham (Buckinghamshire, England) were used for antibody detection (30-90 seconds of exposure) and blots were densiometerically scanned using a Bio-Rad Laboratory imaging densitometer (Hercules, CA).

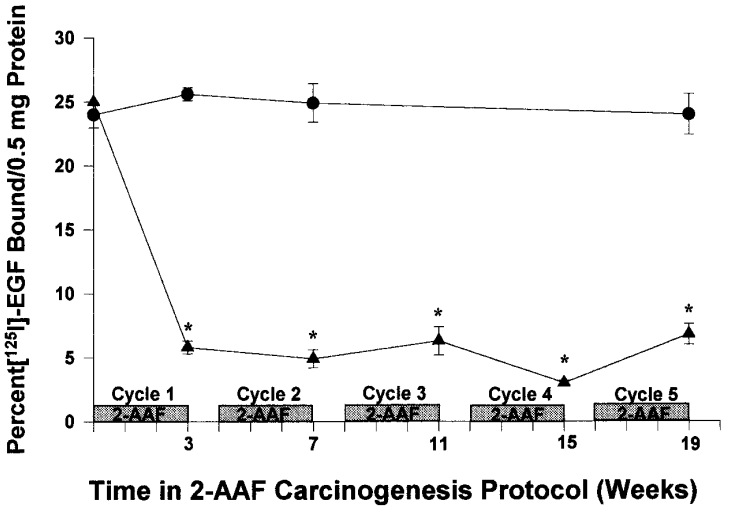

FIG. 1. [^{125}I]-EGF binding levels in microsomes from the livers of rats during 2-AAF-induced hepatocarcinogenesis. EGF binding levels to liver microsomes were determined in rats just completing the 3 wk administration of 0.05% 2-AAF during 1–5 of the cycles (▲). Age-matched control rats were fed basal diet throughout the 19 week protocol (●). Each point represents the mean ± SEM of 4 rats. An * indicates a value which is significantly different ($P < 0.05$) from rats fed basal diets.

RESULTS

[^{125}I]-*EGF Binding Levels of Rats during 2-AAF-Mediated Production of Hepatocarcinogenesis*

Shown in Fig. 1 are the results of studies which monitored EGF binding levels during a 19 wk protocol of cyclic 2-AAF administration. This protocol has been shown to result in 90-100% incidence of hepatic cancer at 12 weeks post 2-AAF administration (12). Levels of EGF binding were found to be decreased to 20-25% of normal levels throughout the 2-AAF dietary protocol and indicated that the down modulation of EGF binding occurred during a period of prolonged carcinogen administration known to induce liver cancer.

Development of a Persistent Loss of [^{125}I]-*EGF Binding Capacity*

Previous studies employing the cyclic administration of 2-AAF to rats have shown that a transition between low to high risk for cancer development occurred between 3 and 4 cycles of 2-AAF administration (11, 12). To evaluate whether the 2-AAF mediated decrease in hepatic EGF binding capacity may be linked to this increased risk for tumor development, the persistence of lowered EGF-binding levels were determined for rats at various points in the 2-AAF protocol following their return to basal diet for 1-3 weeks, see Fig. 2. As shown in Fig. 3, when rats that had completed 1-3 cycles of 2-AAF exposure were fed basal diet for 1-3 weeks, EGF binding levels returned to 80-90% of normal values. In contrast, rats administered 2-AAF for 4 or 5 cycles before being placed on basal diets, recovered to only 30-40% of normal values. Furthermore, rats administered 5 cycles were found to remain at low levels of EGF binding capacity approximately one year (48 weeks) after being returned to basal diet.

Immunochemical Analysis of 2-AAF Mediated Lowering of EGF Receptor Levels in Rat Hepatic Tissues

Immunochemical Western blot analysis of EGF receptor abundance was performed to assess whether 2-AAF caused a loss in EGF receptor number, and if this loss was maintained after

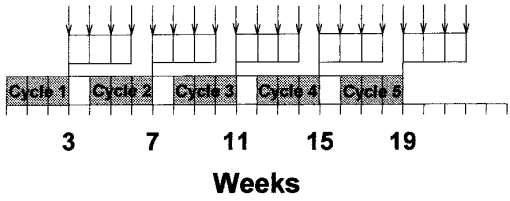

FIG. 2. Dietary protocol for assessing the persistence of 2-AAF mediated decreases in liver EGF binding capacities for rats fed diet with (shaded boxes) or without (clear boxes) 2-AAF. Arrows are sampling points for determining hepatic EGF binding levels.

4 or more cycles of 2-AAF. Results showed that plasma membranes from the livers of rats fed 2-AAF for 3 or 5 cycles had EGF receptor levels that were significantly reduced, 31.1 ± 7% and 40.4 ± 5% respectively, of the levels of EGF receptor found in age-matched controls, Fig. 4. But after a 3 week recovery period, in which the rats were fed the semi-synthetic control diet alone, the rats completing 3 cycles of 2-AAF administration showed an increase to 101.9 ± 29.8% of control rats, while the rats completing 5 cycles of 2-AAF remained low, at 31.3 ± 3% of control.

DISCUSSION

These studies have evaluated the ability of 2-AAF, a complete hepatocarcinogen, to mediate a lowering of rat liver EGF binding capacity and receptor protein during early and intermediate stages of hepatocarcinogenesis. They showed that 2-AAF mediated a persistent loss in EGF binding, and EGF receptor protein level, during a transition from low to high risk for progression to liver cancer.

Rats have previously been shown to undergo a transition from low to high risk for the development of hepatocarcinomas between 3 and 4 cycles of 2-AAF exposure (12). In addition, previous studies from this laboratory have shown that carcinogenesis-related irreversible alter-

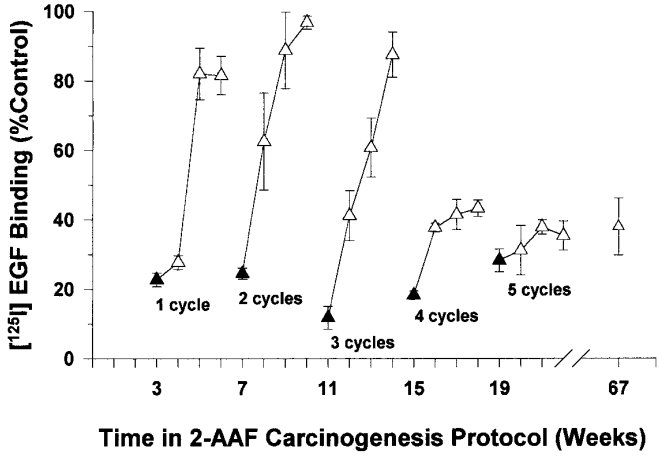

FIG. 3. Recovery of [^{125}I]-EGF binding capacity following removal of rat from 2-AAF diet. EGF-binding levels to liver microsomes were determined immediately after (▲), and at 1, 2, and 3 weeks post 2-AAF administration (△) for each of the 5 cycles, and at 67 weeks (48 weeks post 2-AAF administration) for rats completing 5 cycles. Age-matched control rats were fed basal diet throughout the duration of the experiment. Values represented are percent of controls ± SEM for 4 rats/point.

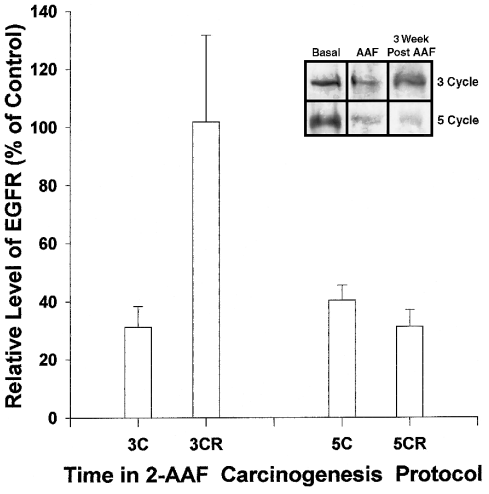

FIG. 4. Immunochemical analysis of EGF receptor protein levels in livers of rats subjected to 2-AAF treatment. Hepatic plasma membranes isolated from control rats fed basal diet, from rats immediately following 3 or 5 cycles of 2-AAF exposure (3C or 5C, respectively), and from rats following 3 weeks recovery on basal diet after 3 or 5 cycles of 2-AAF administration (3CR or 5CR , respectively), were evaluated immunochemically with monoclonal antibody for EGF receptor. The inset shows representative Western-blot (50 μg/lane) immunochemical staining for EGF receptor. The relative EGF receptor content for each value is the mean $\pm$ standard error for 3-4 different samples. All values except for 3CR were significantly ($P<0.05$) less than values for control rats fed the basal diet.

ations in hepatic *N*-hydroxy-2-AAF sulfotransferase activity, and the DNA repair related ADP-ribose polymer production, occurred between the 3rd and 4th cycles of 2-AAF administration (12, 17). Thus, the persistent decrease in EGF binding levels observed between 3 and 4 cycles appears to be cancer-stage dependent in that it is associated with reaching a high risk stage in the progression of heptocarcinogenesis. Support for a decrease in EGF binding capacity at the later stage of hepatocarcinogensis was previously reported in studies which showed a persistent decrease in EGF binding in hepatocarcinomas, relative to peritumorous tissue, in rats fed chemical carcinogens and allowed to recover for up to one year on control diet without carcinogen (18). Observations in this laboratory using the 2-AAF protocol reported here, also indicate a decrease in EGF binding capacity by tumor tissues (unpublished results).

The observation that rats removed from the 2-AAF diet during early stages (cycles 1-3) of 2-AAF-induced hepatocarcinogenesis recovered EGF binding capacity to near normal levels, suggests that recovery could be mediated by clearance of cytotoxic 2-AAF metabolites. The persistent decrease in EGF binding capacity may correspond with cancerous cells reaching a stage of autonomous growth which no longer requires EGF stimulation for growth. The above findings indicate that the appearance of a persistent decrease in EGF binding may be a useful biomarker for key stages in hepatocarcinogenesis.

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