ABSTRACT

Experimental chemical hepatocellular carcinomas that were induced in male F344 rats using three different regimens of limited exposure to the carcinogens 2-acetylaminofluorene or diethylnitrosamine were characterized by very low (as compared to peritumorous or normal tissues) binding of epidermal growth factor and decreased autophosphorylation of the epidermal growth factor receptors. Similar changes were also found in the insulin receptors. We suggest that the carcinogens 2-acetylaminofluorene and diethylnitrosamine cause an initial chemical effect on the great majority of cells. Most of them with time recover their receptor function, and only a small minority become truly initiated and retain these changed characteristics up to the tumor stage. The observed changes appear to be associated with the altered growth state induced by chemical carcinogens. Simultaneous changes observed in the two receptors raise the possibility of a common underlying mechanism.

INTRODUCTION

The dietary administration to rats of the hepatocarcinogen AAF results in an early and sharp decrease in the binding of EGF and insulin to their corresponding receptors in liver microsomal and Golgi fractions (1, 2). Qualitatively similar effects are caused also by DEN (3). After the administration of the carcinogens is stopped, the length and completeness of recovery depend on the duration of the preceding carcinogen administration.

We reasoned that, if the observed changes were explained only by the acute hepatotoxicity of the carcinogens, they should have been restricted to the period of administration of the carcinogens and immediately following it. If, on the other hand, the changes are related to the carcinogenic process, they should persist in the initiated cells and would find their expression in the resultant hepatocellular carcinomas. To answer this question, we investigated the binding of EGF and insulin to the liver Golgi fraction of tumors and peritumorous tissues of rats that developed hepatocellular carcinomas 1 yr after the administration of the carcinogens.

Persistent markers of carcinogenic changes, if and when found, would facilitate the research of several important aspects of the transformation process. We suggest that, in the specific case of AAF- and DEN-induced hepatocellular carcinomas, changes in the EGF and insulin receptors expressed as low binding and receptor autophosphorylation represent such markers.

MATERIALS AND METHODS

Animals. The experiments were conducted on male F344 rats (180-200 g wt). For the production of tumors we used three regimens: (a) the Solt-Farber schedule, a single initiating dose of DEN (200 mg/kg) followed 2 wk later by 2 wk of feeding of 0.02% AAF, and by a partial hepatectomy after the first wk of AAF; (b) chronic administration of 0.02% AAF in the food for a period of 3 mo followed by a return to a basal diet; and (c) a single dose of DEN (200 mg/kg) 24 h after a 1/2 hepatectomy followed 1 mo later by the feeding of 0.05% phenobarbital continuously for 11 mo. Age-matched control rats were fed a basal diet (Purina laboratory Chow) for 1 yr.

Rats were sacrificed by exsanguination under light ether anesthesia at the age of 1 yr. Their livers were perfused in situ with cold 0.9% NaCl solution; the tumors were separated as thoroughly as possible from the peritumorous tissues. The separation was inevitably incomplete; whereas the "tumors" contained almost exclusively hepatocellular carcinomas, what we call "peritumorous tissues" inevitably contained also a certain amount of tumorous cells. In the rats, the livers of which were used for the insulin competition curves, noncarcinomatous tissue could not be separated with certainty.

Tissue Preparation and Assays. The preparation of the Golgi fractions and the binding assays were conducted as described (1, 2, 4). The Golgi nature of the appropriate fraction was confirmed by electron microscopy and galactosyltransferase enzyme marker. The binding was calculated as the percentage of specific binding per 100 g of protein. Autophosphorylation was determined according to Fernandez-Pol (5) and Kasuga (6) in the following way. Receptors were preincubated with or without insulin or EGF in concentrations of 10^{-10} M. The concentration that was optimal in our experiments was 10^{-4} M, and it was used throughout the study. We found the cross-phosphorylation of EGF-stimulated insulin receptors or insulin-stimulated EGF receptors to be less than 10%; therefore we included both ligands in the receptor autophosphorylation experiments. The reaction was conducted with 10 µg of protein of partially purified receptors (in 100 µl) with 100 µl of buffer containing 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM MgCl₂, 10 mM MnCl₂, and bovine serum albumin (1 mg/ml), pH 7.4. The mixture was preincubated with or without the ligands (1 µM) at 22°C for 30 min. Receptor autophosphorylation was initiated by the addition of 20 µCi of [γ-32P]ATP, 10-20 Ci/mmol (ICN Radiochemicals, Irvine, CA). After an incubation for 10 min at 22°C, phosphorylation was terminated by the addition of 50 µl of sample buffer [313 mM Tris-HCl (pH 6.8)-10% sodium dodecyl sulfate-0.05% bromophenol blue-50% glycerol-0.5 M dithiothreitol]. The samples were boiled for 5 min, and aliquots of 120 µl were loaded onto a 4% polyacrylamide gel containing 7% acetic acid, dried in a vacuum, and autoradiographed at -70°C for 24 h using Kodak X-AR film and a Dupont Cronex lighting-plus intensifying screen. The corresponding bands of EGF (M, 170,000) and insulin (M, 95,000) receptors were excised from the dried gels, and the radioactivity of 32P was measured in a liquid scintillation counter.

We found significant day-to-day variability in the binding, and some decrease in the binding of EGF and insulin with ageing; therefore three control 1-yr-old rats were always sacrificed on the same day. In view of the non-normal distribution of the results, statistical comparisons were done by the nonparametric sign test, and the correlation was calculated as Spearman p values (8).

RESULTS

The results of the binding experiments are presented in Fig. 1. Although there was no qualitative difference between the tumors induced by various methods, the binding of both ligands

1 The work was made possible in part by Grant CA35572 from the National Cancer Institute.

2 The abbreviations used are: AAF, 2-acetylaminofluorene (N-fluoren-2-yl-acetamide); EGF, epidermal growth factor; DEN, diethylnitrosamine (N-ethyl-N-nitrosourea); TGF, transforming growth factor(s).
was slightly lower in rats treated continuously with AAF. As can be seen (Fig. 1), in some cases the binding of the ligands to their corresponding receptors in the peritumorous tissues was higher than in the control livers.

In the control 1-yr-old rats the median specific binding of EGF to the Golgi fraction of the liver was 8.1% (range, 2.0–16.6%). The median binding to the tumors was 1.7% (range, 0.3–4.6%) and to the peritumorous tissues, 5.8% (range, 1.2–12.7%). In all cases the binding of EGF to the tumors was much lower than to the peritumorous tissues ($P < 0.001$), but there was a strong positive correlation between the binding to the tumors and the peritumorous tissues ($r = 0.819; P < 0.001$). The competition curve (Fig. 2) showed that the observed decrease in tumors was due to the drop in the number of receptors ($R$) with a possible small increase in their affinity. The corresponding values were: for controls, $R_0$ 5.4 pmol/mg, $K_d$ 3.80 nM; for peritumorous tissue, $R_0$ 4.0 pmol/mg, $K_d$ 4.37 nM; and for the tumors, $R_0$ 1.1 pmol/mg, $K_d$ 3.04 nM. The autophosphorylation of the EGF receptors (Fig. 4) as reflected by $^{32}P$ incorporated into the $M_r$ 170,000 band, in comparison to controls, was: in tumors unstimulated by the ligand, 16–44%; and stimulated, 21–41%; in the peritumorous tissues correspondingly, 29–99% and 37–94%. In all cases the autophosphorylation of the EGF receptors in tumors was only 36–65% of that found in the peritumorous tissues of the same rats.

The median specific binding of insulin to the livers of 1-yr-old rats was 7.7% (range, 3.5–13.3%). The binding to tumors was only 3.8% (range, 1.3–8.7%), and the binding to peritumorous tissues, 6.7% (range, 1.9–9.3%); the difference was highly significant ($P = 0.007$), but the bindings to the tumors and peritumorous tissues did not show significant correlation ($r = 0.314; P = 0.350$). The competition curve (Fig. 3) showed that the decreased binding to tumors was due to the significant drop in the number of receptors with some small increase in
BINDING OF EGF AND INSULIN IN RAT HEPATOCELLULAR CARCINOMAS

Fig. 4. Phosphorylation of the insulin and EGF receptors in hepatocellular carcinomas and peritumorous tissues.

<table>
<thead>
<tr>
<th>Animals and treatment</th>
<th>Unstimulated</th>
<th>Stimulated</th>
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<tbody>
<tr>
<td></td>
<td>$M$, 95,000</td>
<td>$M$, 170,000</td>
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<tr>
<td>12-mo-old rats</td>
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<td>AAF</td>
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<tr>
<td>Peritumor</td>
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<td>(A) 1363</td>
</tr>
<tr>
<td>Tumor</td>
<td>(C) 312</td>
<td>(C) 601</td>
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<tr>
<td>DEN + phenobarbital</td>
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<td></td>
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<tr>
<td>Peritumor</td>
<td>(D) 193</td>
<td>(D) 405</td>
</tr>
<tr>
<td>Tumor</td>
<td>(E) 162</td>
<td>(E) 266</td>
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<tr>
<td>Solt-Farber</td>
<td></td>
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<tr>
<td>Peritumor</td>
<td>(F) 232</td>
<td>(F) 601</td>
</tr>
<tr>
<td>Tumor</td>
<td>(G) 179</td>
<td>(G) 223</td>
</tr>
<tr>
<td>3-mo-old rats</td>
<td>(H) 398</td>
<td>(H) 1313</td>
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</tbody>
</table>

* Letters in parentheses, lanes.

their affinity: for controls, $R_1$ 2.4 pmol/mg, $K_1$ 1.92 nM, and $R_2$ 3.3 pmol/mg, $K_2$ 3.30 nM; for the tumors, $R_0$ 0.5 pmol/mg, $K_0$ 1.43 nM. The autophosphorylation of the $M$, 95,000 band (insulin receptor) as compared to the control values was (Fig. 4): in the tumors unstimulated by insulin, 44–85%; stimulated, 24–51%; and in the peritumorous tissues, correspondingly, 53–99% and 29–65% of the control values. In all cases the autophosphorylation of the receptors in tumors was only 73–85% as high as in the peritumorous tissues.

DISCUSSION

Our results show that hepatocellular carcinomas developing a long time after the administration of carcinogens are characterized by low EGF and insulin binding and receptor autophosphorylation. In all instances the contrast between the tumors and the surrounding tissues was pronounced.

It is hardly probable that the changes found in the tumors were the consequence of the toxicity of the carcinogens administered a long time before: after the acute administration of DEN, the binding of insulin and EGF sharply decreases but rebounds to the initial level by 30 days (3). In addition, had the effect been general toxicity, we would expect it to persist in the peritumorous tissues, whereas we found the binding to the peritumorous tissues to be rather close to one observed in the control animals. Consistent differences in the autophosphorylation of the partially purified receptors in tumors and peritumorous tissues also could hardly be explained by the toxic effects of the chemicals. Therefore it seems much more likely that, whereas in the short run, AAF and DEN affected all or the great majority of the liver cells, in the long run, only a small minority of them (initiated?) preserved these new characteristics and passed them to further generations of cells during tumorigenesis. Since not only EGF binding but also its receptors’ autophosphorylation was decreased in tumors, it is probable that the hepatocellular carcinomas were characterized by the decreased expression of the receptors and not only by the changes in their ligand-binding site.

Both EGF and insulin receptors have extensive homology to certain oncogene proteins (9–11). It has been recognized that cell transformation can result in growth factors’ autonomy of cancer cells through alterations of receptors or postreceptor signaling rather than through the altered production of growth factors themselves (12). However, most discussion centered around the phenomenon opposite to one found by us, namely, the increased expression of the EGF receptors in certain tumors, e.g., in many human gliomas (13), bladder cancers (14), and breast cancers (14, 15). On the other hand, in astrocytomas, meningiomas (13), hepatocellular carcinomas (16, 17), and in some breast and bladder tumors (14, 15) and gliomas (13), the EGF receptors were very low or not identified. The loss of EGF receptors was reported in chemically or spontaneously transformed liver cells in vitro (18), and concomitant with this, receptor autophosphorylation was also decreased (19). Since even normal cell types demonstrate differences in EGF receptors (20), the same can be logically expected in different lines of tumors. It is probable that, in those tumors that demonstrate decreased EGF binding, EGF becomes either superfluous or even detrimental to the autonomous growth; e.g., EGF suppresses the primary culture of human hepatoma xenograft (21). So, in different tumors EGF receptor levels can be increased, decreased, or not changed, as reviewed recently (22). It would be logical to suggest that low EGF binding could be the result of TGF-α production by the cells and that such an interaction...
of TGF-α with their receptors could take place in the Golgi system. But in this case we would expect the coexistence of decreased EGF binding with the stimulation of the EGF receptors’ autophosphorylation which was not the case.

In relation to insulin receptors, the experimental hepatocellular carcinomas showed significant differences: HTC cells had 6 times less receptors than H35 cells (23), and insulin binding was directly proportional to the degree of differentiation (24, 25). Zajdela rat ascites hepatoma cells showed a decreased number of insulin receptors (26). Depression of the insulin receptors cannot be explained by the production of TGF-α.

Both EGF and insulin receptors belong to the same class as certain oncogene proteins, characterized by autophosphorylation and tyrosine kinase activity. Growth hormone receptors, though, do not belong to this class, and a decrease in the growth hormone binding to hepatomas can point to the effects of AAF and DEN on wider categories of receptors than heretofore considered (27). The simultaneous changes in several receptors point to a common underlying mechanism, probably transcriptional in nature.

The changes in the EGF and insulin binding are pronounced and deserve further study as possible markers of the carcinogenic process in the liver. These markers can hardly be unique; e.g., it has been shown that, after the administration of AAF or DEN, both hyperplastic nodules and hepatomas contained increased amounts of several peptides, including glutathione S-transferase (28). It is possible that the observed changes in the receptors are peculiar for these types of hepatomas and that only some of the multiple effects of the chemical carcinogens are directly related to carcinogenicity. Irrespective of whether the changes in the expression of the receptors are pathogenetically related to carcinogenesis or are only coincidental markers of certain tumors, the study of them can be of significant value for better understanding of the chronological development of hepatomas.

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Binding of Epidermal Growth Factor and Insulin and the Autophosphorylation of Their Receptors in Experimental Primary Hepatocellular Carcinomas


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