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HEPATOCARCINOGENS INDUCE DECREASE IN mRNA TRANSCRIPTS OF RECEPTORS FOR INSULIN AND EPIDERMAL GROWTH FACTOR IN THE RAT LIVER

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Gene expression of the receptors for insulin and epidermal growth factor (EGF) was studied in the livers of rats after a single injection of a hepatocarcinogen diethylnitrosamine (DEN) 100 mg/kg or after feeding the animals N-acetylaminofluorene (AAF) 0.02% w/w. DEN induced a time-dependent decrease in mRNA transcription of the receptors for insulin (10.3 and 8.5 Kb) and EGF (10.0, 5.8 and 2.8 Kb), evident already after 4 hours, reaching a nadir of 10-20% of the initial level between 16 and 24 hours and returning to normal by 10 days. In rats fed AAF, transcription of both receptor genes decreased to less than 20% of the control values after 2 days. In the livers of rats treated with DEN, that developed hepatocellular carcinomas one year later, expression of both receptors was also very low. DNA showed no changes. The results suggest that the hepatocarcinogens or their metabolites decrease RNA transcription or destabilize the steady state RNA level of both receptors in the early phase of toxic effect and that some tumor-derived products may be involved in the same phenomenon in the later stage of tumor development.

Hepatocarcinogens N-acetylaminofluorene (AAF) and diethylnitrosamine (DEN) cause drastic reduction in the number of insulin and EGF receptors in the livers of rats (1-4). In the case of AAF feeding, the number of both receptors decreases sharply already after 2 days and remains low for the whole 3 month period of continuous AAF exposure. In the case of a single injection of DEN, a sharp drop in the receptors is followed by their recovery. Hepatocellular carcinomas that develop one year later are also characterized by very low expression of insulin and EGF receptors (5).

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To study the mechanism of these changes, we used the Northern blot technique of hybridizing total RNA of the rat liver with the DNA probes of insulin and EGF receptors. The results reported below show that the likely explanation of the decrease in the number of both receptors is reduction of their mRNA transcription.

MATERIALS AND METHODS

Animals. Male F-344 rats (180-200 g) (Charles River Laboratory, Wilmington, MA) were housed under the standard conditions of temperature and humidity and 12 hour light-darkness cycle. The animals had unlimited access to water and food (Purina Lab Chow) and were maintained according to the NIH Guide for the Use of Laboratory Animals. The groups of rats were sacrificed by exsanguination under light ether anesthesia, their livers were perfused in situ with saline to remove most of the blood, and RNA was extracted.

Treatment schedules. Two hepatocarcinogens were used. DEN was administered as a single i.p. injection (100 mg/kg). The rats were sacrificed after 4h, 16h, 24h, 36h, 48h and 10 days. In another series AAF was added to food 0.02% w/w, and the animals were sacrificed after 2, 7 and 14 days. Some animals injected once with DEN were sacrificed at the age of one year when they developed hepatocellular carcinomas. In all the experiments age-matched control rats were sacrificed on the same day. Triplicate experiments were performed for each treatment.

RNA extraction and quantitation of receptor gene transcripts. Total cellular RNA was isolated with guanidine/cesium chloride method (6). After electrophoresis through a 1% agarose gel containing 6.6% formaldehyde, RNA was blotted (Northern blot) onto nylon membrane (ICN Radiochemicals, Irvine, CA). The probe for for EGF receptor was a human 1.8 Kb 5'-end EcoRI DNA fragment (7). The insulin receptor probe was a subclone of 4.1 Kb EcoRI fragment of human insulin receptor DNA (8). Both probes were labelled with [^{32}P] by nick-translation to a specific activity 2-3 x 10^8 cpm/µg. The nylon membrane was prehybridized at 42°C for 1 hour with buffer containing 50% formamide, 5 x Denhardt's solution, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 200 µg/ml calf thymus DNA and 100 µg/ml yeast tRNA. Then the membrane was hybridized with the probe (2 x 10^7 cpm in 8 ml buffer) at 42°C for 48 hours with the same buffer but using 1 x Denhardt solution and 20 mM sodium phosphate pH 6.5. The membrane was washed for 10 minutes four times with 2 x SSC - 1% SDS at room temperature and then for 15 minutes twice with 0.2 x SSC - 0.1% SDS at 45°C. The air dried membrane was exposed to Kodak XRP-5 x-ray film at -70°C with an intensifying screen for 3 days.

DNA extraction, digestion with restriction endonucleases and Southern blotting. Total cellular DNA was isolated from the nuclei of the liver cells (9). DNA was digested with restriction endonucleases HpaII, MspI, BamHI, EcoRI, HindIII and PstI under the appropriate conditions and blotted to nylon membrane

(6). The membrane was then hybridized with nick-translated [^{32}P]-DNA probes for insulin and EGF receptors.

RESULTS

Figure 1 represents the RNA transcription of the EGF (panel A) and insulin (panel B) receptors in the livers of control and AAF-treated rats. Three different sizes of mRNA (10.0, 5.8 and 2.8 Kb) hybridized to the EGF receptor probe. The 28S rRNA region also showed some cross-reactivity. Two RNA bands (10.3 and 8.5 Kb) were detected with the insulin receptor probe. Both EGF and insulin receptor RNA levels were sharply reduced after 2 days of AAF treatment. An equal amount of RNA was loaded as judged by the similar intensity of ribosomal RNA bands on agarose gel stained by ethidium bromide and by probing the same transfer blot with β-actin cDNA probe (data not shown).

Figure 2 shows the time course of the RNA expression after a single injection of DEN 100 mg/kg. The EGF receptor RNA transcripts (Fig. 2A) began to decrease already 4 hours after the injection and reached their nadir at 16 hours after which they recovered and reached the initial value by 10 days.

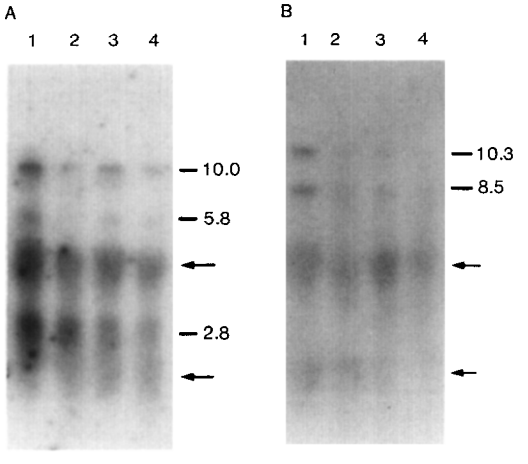

Figure 1. Northern blot of rat liver total RNA (20 µg/lane) hybridized with [^{32}P] -labeled EGF receptor DNA probe (panel A) and with insulin receptor DNA probe (panel B). Lines: 1 - two-month-old control rat; 2 - rat fed AAF for 2 days; 3 - same for 7 days; 4 - same for 14 days. RNA size markers are 9.5, 7.5, 4.4, 2.4 and 1.4 Kb (BRL Corp., MD). Arrows indicate 28S (4.7 Kb) and 18S (1.9 Kb) respectively.

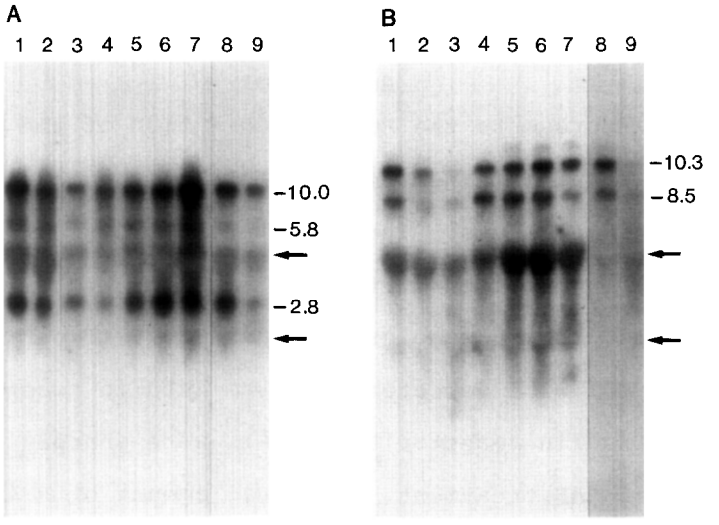

Figure 2. Northern blot of rat liver total RNA (30 µg/lane) hybridized with [^{32}P]-labeled EGF DNA probe (panel A) and with insulin receptor DNA probe (panel B). Lanes: 1 - two-month-old control rat; lanes 2-7 - rats injected with 100 mg/kg DEN and sacrificed after 4, 16, 24, 36, 48 hours and 10 days, respectively. Lane 8 - one-year-old control rat; lane 9 - rat injected with DEN 100 mg/kg and sacrificed one year later. RNA size markers and arrows - as in Figure 1.

However, the receptor transcription was very low (about 20% of the control) in the livers of those rats that had developed multiple neoplastic or preneoplastic nodules one year later (Fig. 2A, lane 9). The dynamics of the changes in the insulin receptor RNA transcript were almost the same as in the case of EGF receptors. In the nodular liver, however, insulin receptors mRNA transcripts were lower than those of EGF receptors (Fig. 2B, lane 9).

Six restriction endonucleases (HpII, MspI, BamHI, EcoRI, HindIII and PstI) were used to digest the DNA isolated from the rat livers. No changes were observed in animals treated with either carcinogen (data not shown).

DISCUSSION

Both insulin and EGF are known liver mitogens. Receptors for both of them have intrinsic tyrosine kinase activity, and both ligands exert strikingly similar effects on rat hepatocytes

(10). Therefore to study the regulation of both seemed logical and philogenetic conservation of the receptors made the use of human probes possible.

Changes in the EGF and insulin binding to different tumors have been repeatedly reported. In most cases, however, interest centered on the increased expression of the EGF receptors, even when it was found only in a small number of certain tumors. On the other hand, working with experimental hepatocarcinogenesis, we found that the binding of both EGF and insulin was not increased but on the contrary, sharply decreased (1-5). These changes were observed both in the acute experiments after a limited time exposure to the carcinogens and in the tumors which developed one year later. The changes in the acute experiments involved the whole organ whereas the initiation of the tumor takes place in only a small minority of cells; the changes observed in the one-year-old rats with tumors, however, demonstrated significant differences between the tumorous and non-tumorous parts of the liver. Therefore the mechanism of the acute changes and changes observed in the tumors would not seem to be necessarily the same. For that reason we undertook the present study. Theoretically, a decrease in the number of receptors could be explained by the DNA modification, mRNA transcription, mRNA turnover, translation of the receptors or their post-translational activation and translocation. We used specific DNA probes for the analysis of cellular total RNA, and the results clearly showed that in the AAF-treated rats the expression of RNA transcripts was sharply reduced after 2 days and remained low thereafter, the results consistent with the decreased binding of the two ligands to the microsomal and Golgi fractions of the livers of these rats (1-5). In the animals treated with the single injection of DEN, the changes went through several

phases: a sharp initial decrease followed by recovery and the new drop when the animals developed tumors. This, too, corresponds to the changes observed in the binding of the ligands. No changes in DNA were found.

Little is known about the transcription of mRNA for the EGF receptors. In the human pancreatic carcinoma cells no DNA amplification was found but mRNA level correlated with the EGF receptors number (11). In human primary squamous cell carcinomas with high level of EGF receptors, researchers found in some cases gene amplification (12,13), whereas in the placental tissue higher level of mRNA could be generated by a greater mRNA stability and/or transcription (14). On the other hand, in many tumors increased number of EGF receptors was not accompanied by the changes in either DNA or mRNA (15). In no available publication could we find the explanation of the decreased number of EGF receptors, and no information at all concerning the mechanism of the changes in the number of the insulin receptors.

The results of the present study show that decreased binding of EGF and insulin to the liver tissues after the application of the two hepatocarcinogens and their decreased binding to the hepatocellular carcinomas have the same underlying mechanism, i.e. decreased level of mRNA. Whether this regulatory process is directly or only tangentially related to hepatocarcinogenesis, remains to be seen. In any case, the changes in the transcription showed striking parallelism, especially intriguing in view of the different localization of the genes for insulin and EGF receptors. This raises the intriguing question of the possible common underlying mechanism.

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