

Expression and inducibility of drug-metabolizing enzymes in preneoplastic and neoplastic lesions of rat liver during nitrosamine-induced hepatocarcinogenesis

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Abstract

The expression, inducibility, and regulation of four different cytochrome (cyt.) P-450 isoenzymes (PB₁, PB₂, MC₁, and MC₂), NADPH-cytochrome P-450 reductase, the glutathione transferases (GSTs) B and C and microsomal epoxide hydrolase (mEH_b) have been studied during nitrosamine-induced hepatocarcinogenesis using immunohistochemical techniques. The investigations revealed basic differences in the expression of the individual drug metabolizing enzymes in the course of neoplastic development. While the two GSTs and mEH_b were increased in all preneoplastic and benign neoplastic lesions, the levels of the distinct cyt. P-450 isoenzymes were characteristically different from each other. Following initial changes in the expression of these enzymes in early preneoplastic lesions (i. e., increase of cyt. P-450 PB₁, versus slight decrease of the other cyt. P-450 isoenzymes), a continuous reduction of all cyt. P-450 isoenzymes was observed during the further course of hepatocarcinogenesis. In progressed neoplastic nodules, all cyt. P-450-isoenzymes and NADPH cyt. P-450 reductase were decreased to varying extents.

Treatment of animals with inducers of the monooxygenase system, such as phenobarbital, 3-methylcholanthrene and polychlorinated biphenyls, led to a rather heterogenous pattern of enzyme alterations in preneoplastic and neoplastic lesions. Following administration of phenobarbital, some islets responded to the same degree as the surrounding tissue, others were less or not at all inducible and a few of the lesions showed a prominent increase in cyt. P-450 PB₂ and NADPH-cyt. P-450 reductase levels. The interesting finding that these two enzymes always showed concurrent changes may be indicative of a common regulation. Similar to phenobarbital, an induction of cyt. P-450 isoenzymes within carcinogen-induced lesions was also observed following administration of 3-methylcholanthrene or polychlorinated biphenyls.

The results demonstrate that drug-metabolizing enzymes are abnormally regulated in carcinogen-induced lesions. The multiplicity of enzyme deviations within individual lesions and especially the enzyme inducibility strongly suggest that the focal enzyme alterations result from genotoxic effects of the carcinogen on regulatory systems of a higher order rather than from mutational events in individual structural genes.

Key words: Drug-metabolizing enzymes – Liver – Rat – Hepatocarcinogenesis

Introduction

Chemically-induced hepatocarcinogenesis is known to be a multi-step process which is associated with the early appearance of focally growing, presumably preneoplastic cell populations (Emmelot and Scherer 1980; Bannasch et al. 1980; Farber 1980). These cells are characterized by multiple alterations in their phenotype which have been studied on the basis of biochemical, morphological and histochemical properties. A common feature of preneoplastic and neoplastic cells are abnormalities in the expression of drug-metabolizing enzymes which play an important role in the metabolism of both endogenous and exogenous substrates (Conney 1967; Gillette et al. 1972). Enzymes such as microsomal epoxide hydrolase (Enomoto et al. 1981, Kuhlmann et al. 1981), different glutathione transferases (Tatematsu et al. 1985), and UDP-glucuronyl transferase (Bock et al. 1982), which are largely involved in detoxifying processes, are increased in preneoplastic and benign neoplastic lesions. In contrast, the preferentially carcinogen-activating microsomal monooxygenases have been found to be decreased in hyperplastic nodules (Cameron et al. 1976; Okita et al. 1976; Aström et al. 1983) and hepatomas of different growth rate (Sugimura et al. 1966). Individual cytochrome P-450 isoenzymes, however, may also be increased in early preneoplastic lesions (Buchmann et al. 1983, 1985; Schulte-Hermann et al. 1984).

The cellular mechanisms which are responsible for the observed enzyme alterations in carcinogen-induced lesions are still unclear. In view of the known genotoxic effects of carcinogens (Magee et al. 1975), it appears likely that mutational events in both individual structural genes and regulatory genes of a higher order may affect the regulation and expression of enzymes, thus leading either directly or indirectly to an enhancement or repression of enzyme synthesis. In order to further elucidate the regulatory principles operable in preneoplastic and neoplastic cells, we investigated the expression and inducibility of different cytochrome (cyt.) P-450 isoenzymes, NADPH-cyt. P-450 reductase, microsomal epoxide hydrolase (mEH_b) and the glutathione transferases (GSTs) B and C during the process of carcinogen-induced hepatocarcinogenesis using immunohistochemical techniques.

Materials and methods

Purification of protein and preparation of antibodies

Cyt. P-450 isoenzymes (PB₁, PB₂, MC₁ and MC₂), NADPH-cyt. P-450 reductase, the glutathione transferases B and C and the microsomal epoxide hydrolase mEH_b were purified from livers of male Sprague Dawley rats as previously described (Bentley and Oesch 1975; Friedberg et al. 1983; Wolf and Oesch 1983; Wolf et al 1984). The cyt. P-450 isoenzymes are currently being analyzed on the basis of N-terminal aminoacid sequences, which will allow comparison with those forms already described in the literature (Wolf 1986). mEH_b is the microsomal epoxide hydrolase with broad substrate specificity, diagnostically including

benzo[a]pyrene 4,5-oxide (Oesch et al. 1984). Antibodies against these enzymes were prepared in rabbits (Wolf and Oesch 1983).

Immunohistochemical studies

Treatment of animals. Female Wistar rats of about 70 g were obtained from the Zentralinstitut für Versuchstiere (Hannover, FRG) and kept on a standard diet of Altromin pellets (Altromin, Lage, FRG) and water with a light and dark cycle of 12 h each. Diethylnitrosamine (DEN) was administered at a dose level of 100 ppm in the drinking water for 10 days, and N-nitrosomorpholine (NNM, 100 ppm in drinking water) was given chronically for 35 weeks. Following the carcinogen dosing, groups of animals were treated with various inducers of the microsomal monooxygenase system. Phenobarbital (PB) was given either chronically (500 ppm) in the diet or on 3 consecutive days (80 mg/kg) prior to sacrifice of the animals. 3-Methylcholanthrene (3-MC) was injected IP (40 mg/kg, in corn oil) on 3 consecutive days prior to sacrifice and polychlorinated biphenyls (PCBs) were administered IP (150 µmol/kg, in corn oil) once weekly over a period of 8 weeks.

Enzyme and immunohistochemistry. Animals were sacrificed under ether anesthesia. Livers were carefully removed and immediately frozen at -40 °C. Serial sections (10 µm) of the large median lobe were prepared on a cryostat microtome and used for enzyme and immunohistochemical procedures. Adenosine triphosphatase (ATPase) activity was demonstrated according to the method of Wachstein and Meisel (1957) and γ -glutamyltranspeptidase (γ -GT) activity according to Lojda et al. (1976). Immunohistochemical demonstrations of the different drug-metabolizing enzymes and NADPH-dependent reduction of nitro blue tetrazolium (NBT) were performed as previously described (Buchmann et al. 1985). Glycogen content was demonstrated by the PAS reaction after Hotchkis (1948). The slides were lightly counterstained with toluidine blue.

Materials. Goat anti-rabbit IgG antibody labeled with horseradish peroxidase was obtained from Medac (Hamburg, FRG), 3,3'-diaminobenzidine-tetrahydrochloride (3,3'-DAB) from Polysciences (Warrington, USA). Phenobarbital-Na and 3-methylcholanthrene came from Serva (Heidelberg, FRG). Polychlorinated biphenyls were a kind gift of Dr. L. Robertson (University of Mainz, FRG). Nitro blue tetrazolium was purchased from Merck (Darmstadt, FRG). All other chemicals were of the highest grade available from commercial sources.

Results

Using ATPase deficiency as a marker for identification of carcinogen-induced lesions, we could demonstrate by immunohistochemical methods that the individual drug-metabolizing enzymes were characteristically altered within DEN-induced preneoplastic and neoplastic foci. These enzyme alterations were clearly dependent on the time interval between carcinogen exposure and investigation of the animals and on the state of neoplastic development (schematically summarized in Fig. 1, *cf. to original publication*). In small preneoplastic foci, which appeared within a relatively short period after DEN treatment, cyt. P-450 PB₁ was generally increased, while the other cyt. P-450 isoenzymes were either unchanged or slightly decreased. During the further course of hepatocarcinogenesis, cyt. P-450 PB₁ returned to control levels, cyt. P-450 PB₂ and MC₂ started to decline, and cyt. P-450 MC₁ was markedly depressed. In later appearing, expansively growing nodules, all cyt. P-450 isoenzymes and NADPH-cyt. P-450 reductase were decreased, some down to undetectable levels. In contrast to the differential expression of cyt. P-450 isoenzymes, the two GSTs B and C and mEH_b

were increased in all preneoplastic and benign neoplastic lesions. A rapid decline of these enzymes was observed in glycogen-free, basophilic carcinoma which are frequently characterized by increased AFP levels (Fig. 2, *cf. to original publication*). Usually, the majority of lesions which showed increased or unchanged cyt. P-450 isoenzyme levels were also characterized by an increase in γ -GT activity and by excessive glycogen storage. Reduced glycogen contents, which are characteristic of progressed hepatocellular lesions (Bannasch et al. 1980, 1984), were observed in cyt. P-450 negative nodules and in hepatocellular carcinoma.

Treatment of animals with inducers of the microsomal monooxygenase system, such as PB, 3-MC or PCBs, resulted in a prominent increase of the responding enzymes in normal tissue. In carcinogen-induced lesions, inducer treatment led to a rather heterogenous pattern of enzyme alterations. Following administration of PB some islets responded to the same degree as the surrounding tissue, others were less or not at all inducible and few of the lesions showed a prominent increase in cyt P-450 isoenzyme PB₂ and NADPH-cyt. P-450 reductase (Fig. 3, *cf. to original publication*). This effect appeared both when PB was given continuously in the diet or only on 3 consecutive days prior to sacrifice of the animals. Interestingly, induction of cyt. P-450 PB₂ and NADPH-cyt. P-450 reductase was observed preferentially in nodular lesions which, without inducer treatment, showed decreased levels of these enzymes (see above). The finding that cyt. P-450 PB₂ and NADPH-cyt. P-450 reductase always showed concurrent changes may be indicative of a common regulation of these two enzymes. The GSTs B and C and mEH_b were generally induced by PB to at least the same extent as in the surrounding liver tissue. Following cessation of PB treatment, the levels of the various enzymes returned to control values within 2-3 weeks in both normal and in islet tissue.

As observed in animals treated only with carcinogen, islets in livers of 3-MC-induced animals were characterized by either unchanged or decreased levels of the cyt. P-450 isoenzymes MC₁ and MC₂. This finding might indicate that 3-MC does not induce these enzymes in preneoplastic and neoplastic liver cells. However, since 3-MC leads to an increase of cyt. P-450 isoenzymes in normal surrounding tissue, one would expect that the reduction of cyt. P-450 MC₁ and MC₂ in preneoplastic and neoplastic lesions is more pronounced in livers of 3-MC-treated animals. We therefore determined the relative concentration of cyt. P-450 isoenzymes in preneoplastic islets and neoplastic nodules by semiquantitative microscope photometry of the immunoperoxidase staining. As demonstrated in Fig. 4 (*cf. to original publication*), the relative staining intensity of cyt. P-450 MC₁ and MC₂ in DEN-induced liver nodules is reduced less in 3-MC-treated animals than in animals treated with DEN only. This finding indicates that these enzymes are induced within liver nodules to at least the same extent as in the surrounding normal tissue. In a similar way, an induction of cyt. P-450 isoenzymes was seen in liver foci of animals which were treated with 2,2', 4,4', 5,5'-hexachlorobiphenyl (HCBP), a PB-type inducer of cyt. P-450, or 3,3', 4,4'-tetrachlorobiphenyl (TCBP), a 3-MC-type inducer of cyt. P-450 (data not shown). Due to the relatively slow metabolism of these PCBs, the induction of either group of cyt. P-450 isoenzymes in normal and in islet tissue persisted for at least 9 weeks after cessation of treatment with HCBP or TCBP.

Discussion

The data obtained in the present study extend our knowledge on enzyme regulation during the process of malignant transformation, in that the immunohistochemical technique employed enabled us to investigate the expression and inducibility of several enzymes in very early preneoplastic lesions which are too small to be analyzed by biochemical methods. The

immunohistochemical demonstration of the different drug-metabolizing enzymes revealed that the two GSTs B and C and mEH_b were increased in all preneoplastic and benign neoplastic lesions, whereas the levels of the individual cyt. P-450 isoenzymes were characteristically different from each other. Following initial changes in the expression of these enzymes (i. e., increase of cyt. P-450 PB₁, versus slight decrease of the other cyt. P-450 isoenzymes), a continuous reduction of all cyt. P-450 isoenzymes was observed during the further course of hepatocarcinogenesis. In developed neoplastic nodules, all cyt. P-450 isoenzymes were decreased. This finding confirms previous results on the expression of monooxygenases which were obtained by biochemical analyses of hyperplastic nodules and different hepatoma (Sugimura et al. 1966, Cameron et al. 1976; Okita et al. 1976; Aström et al. 1983).

The observation of a gradual decrease in cytochrome P-450 isoenzyme levels during neoplastic development raises the question as to whether these alterations in enzyme expression are caused by direct genotoxic effects of the carcinogen on structural genes encoding for the specific polypeptide chains. Alternatively, the observed changes could result from mutational events in regulatory systems of a higher order. The various alterations may then be explained by adaptive changes in enzyme synthesis and/or turnover as a result of cellular imbalance in the normal pattern of effectors, e. g., endogenous substrates and metabolites. The multiplicity of enzyme alterations within one and the same cell clone and the ordered sequence of changes in enzyme phenotype with time strongly support the latter assumption. The regularity of this process might be interpreted as being part of a more general regulatory program leading to atypical cell differentiation (Weber et al. 1978; Bannasch et al. 1984; Schulte-Hermann 1985). Our previous studies on the expression and inducibility of drug-metabolizing enzymes in normal liver demonstrated that the different cyt. P-450 isoenzymes are specifically localized within distinct lobular regions of the liver acinus. Upon treatment with various xenobiotics certain groups of cyt. P-450 isoenzymes could be markedly induced even in cells which did not express these enzymes prior to inducer treatment (Wolf et al. 1984). This finding indicates that the expression of certain enzymes in normal cells is not an irreversible quality of differentiation but depends on the suppression and derepression of regulatory components. A similar consideration also applies to preneoplastic and neoplastic cells, since treatment of islet-bearing animals with inducers of the microsomal monooxygenase system like PB, 3-MC or PCBs led to an induction of the corresponding cyt. P-450 isoenzymes in carcinogen-induced lesions which was comparable to that seen in normal tissue. The induction of cyt. P-450 isoenzymes was most pronounced in neoplastic nodules, a type of lesion which is characterized by markedly decreased isoenzyme levels without inducer treatment. This result clearly demonstrates that preneoplastic and neoplastic cells still contain the genetic structures required for cyt. P-450 expression. Thus, the observed decrease in cyt. P-450 expression during development of malignancy appears to result from abnormalities in the function of regulatory genes of a higher order rather than from mutational events in individual structural genes. Whether the alterations in the regulatory state of neoplastic cells are related to cell growth, cell differentiation or other cellular parameters remains to be determined in further studies.

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