Number of Simultaneously Expressed Enzyme Alterations Correlates with Progression of N-Ethyl-N-hydroxyethylnitrosamine-induced Hepatocarcinogenesis in Rats

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Preneoplastic and neoplastic liver cell lesions, induced by EHEN (N-ethyl-N-hydroxyethylnitrosamine) in rats, were investigated to establish the numbers of simultaneously expressed altered enzyme phenotypes within the lesion cells. The lesions were divided into 5 classes on the basis of altered expression in one or more of the following 5 enzymes: glutathione S-transferase placental form, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase, and γ-glutamyl transpeptidase. Class 1 lesions contained cells expressing one altered enzyme. Similarly, class 2, 3, 4 and 5 lesions had cells simultaneously expressing 2, 3, 4, and 5 enzyme alterations, respectively. Four histopathological categories of lesions, ACF (altered cell foci) (274 lesions), HN (hyperplastic nodules) (47 lesions), HCC (hepatocellular carcinomas) (99 lesions) and THC (transplanted hepatocellular carcinomas) (5 lesions) were studied. Proliferation potential was assessed in terms of 5-bromo-2′-deoxyuridine (BrdU) incorporation. The distribution profiles of classes 1 to 5 showed a clear reciprocal change from low class (1 to 2 enzymes) predominance in ACF to high class (4 to 5 enzymes) predominance in HN. Increase of BrdU labeling indices was clearly correlated with progression from HN to HCC. Only a small population of class 5 ACF showed a high BrdU labeling index, indicating particular potential for further development. Thus, the stages of EHEN-induced neoplasia were found to be characterized by gradual increase in the number of altered enzyme phenotypes, with acquisition of proliferative potential being associated with further progression towards malignant conversion.

Key words: Altered enzyme phenotype — Hepatocarcinogenesis — Progression

The preneoplastic and neoplastic lesions that develop during experimental hepatocarcinogenesis display several characteristic morphological, functional and biochemical abnormalities. While a large number of investigators have defined potentially useful markers of liver lesions,[1–9] these markers are clearly not all essential or critical for early or late stages of neoplastic transformation during hepatocarcinogenesis.

The placental form of GST-P[6] has proved to be a particularly useful marker for preneoplastic foci, and its application as an end-point lesion has allowed demonstration of a clear dose-dependent correlation between the results of long-term *in vivo* and medium-term tests for a number of different carcinogens.[5] However, the peroxisomal proliferator group of hepatocarcinogens, such as clofibrate and di(2-ethylhexyl)phthalate, give false-negative results when assessed for GST-P-positive lesions because their associated foci and neoplasms are generally atypical in lacking expression of GST-P or GGT.[8–10] Therefore, for analysis of chemically induced carcinogenesis, the best combination of useful markers should be selected.

The present investigation was aimed at assessing different altered enzyme phenotypes to establish the correlation between the numbers of simultaneously expressed altered enzyme phenotypes within each lesion and progression of hepatocarcinogenesis as monitored by proliferative status. The enzymes GST-P, G6PD, G6Pase, ATPase and GGT were selected for this purpose and BrdU incorporation was employed for assessment of proliferation.[11, 12]

MATERIALS AND METHODS

A total of 40 male 6-week-old Wistar rats (Charles River Japan Inc., Atsugi) were maintained on basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) *ad libitum* and housed in plastic cages in an air-conditioned

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[6] Abbreviations: EHEN, N-ethy-N-hydroxyethylnitrosamine; GST-P, glutathione S-transferase placental form; G6PD, glucose-6-phosphate dehydrogenase; G6Pase, glucose-6-phosphatase; ATPase, adenosine triphosphatase; GGT, γ-glutamyl transpeptidase; ACF, altered cell foci; HN, hyperplastic nodules; HCC, hepatocellular carcinomas; THC, transplanted hepatocellular carcinomas; BrdU, 5-bromo-2′-deoxyuridine.

room at $24\pm2°C$ and $60\pm5\%$ humidity. Preneoplastic and neoplastic liver cell lesions were induced by administration of 0.1% EHEN in drinking water for 4 weeks followed by 16–48 weeks without further insult. Rats were killed at week 20–52 of the experiment. THC were obtained after implantation of induced tumors into nude rats (NIH:rnu/rnu).

To label hepatocytes in S-phase, all animals were subjected to intraperitoneal implantation of minipumps (Alza Corporation, Palo Alto, CA) containing 37.5 mg of BrdU (Sigma Chemical Co., St. Louis, MO) released over a one-week period before killing.

Immediately upon killing, the livers were excised. Slices 4–5 mm thick were cut with a razor blade, immersed in isopentane pre-cooled to approximately $-130°C$ in a liquid nitrogen bath, and stored at $-80°C$ in a deep freezer until use. Serial sections cut at 4 μm were applied for the histochemical demonstration of G6PD (membrane method after Meijer and de Vries[13]), G6Pase, ATPase, GGT activities[14] and for visualizing immunohistochemically the binding of GST-P and BrdU. The sections for immunohistochemical examination were fixed in acetone cooled to $-20°C$, before treatment with anti-GST-P antibody (raised as described previously[1]) at
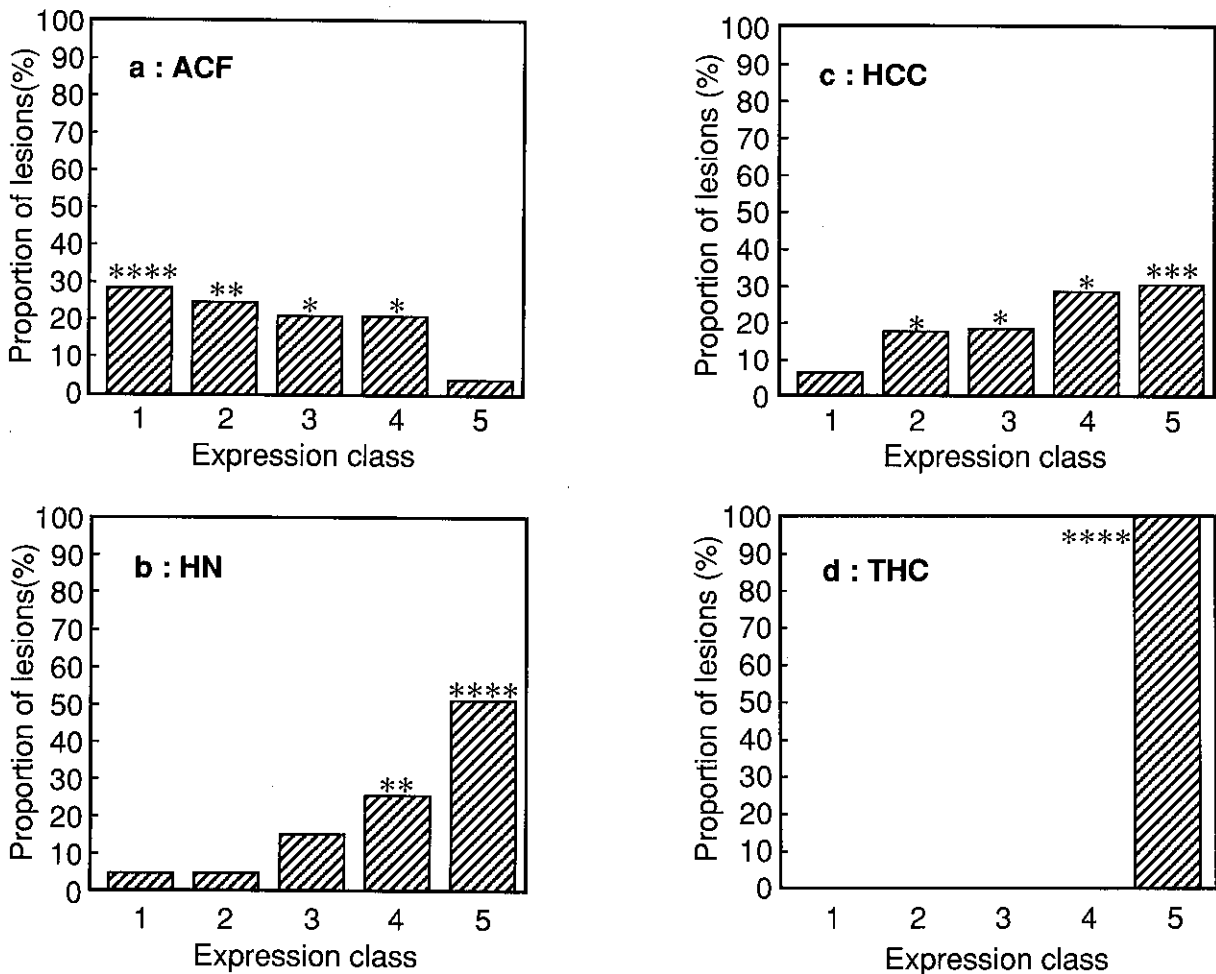

Fig. 1. Lesion classes for a), ACF; b), HN; c), HCC; d) THC. Significantly greater values than for the other 4 (****), 3 (***), 2 (**) or 1 (*) higher expression classes in ACF lesions. Similarly, significantly greater values than for the other 4 (****), 3 (***), 2 (**) or 1 (*) lower expression classes in HN, HCC and THC lesions ($P<0.001$–0.05, chi-square test). Comparison of the 4 histopathological categories using the Mann-Whitney test demonstrated HN to have significantly increased classes over ACF and HCC ($P<0.001$ and $P<0.01$, respectively), HCC demonstrated a significant increase as compared to ACF ($P<0.001$) and THC were significantly increased over both ACF and HCC ($P<0.001$ and $P<0.05$, respectively).

a dilution of 1:6,000 or anti-BrdU monoclonal antibody (Becton-Dickinson Monoclonal Center, Mountain View, CA) at a dilution of 1:200.[11, 12] Binding sites were demonstrated by the avidin-biotin peroxidase complex (ABC) method using diaminobenzidine-H_2O_2[15] and sections were lightly counterstained with hematoxylin.

For the study of altered enzyme expression, individual lesions demonstrating the respective enzyme alteration in more than half of the focal lesion area were traced and overlaid on a sheet of paper using a Microfiche Plaque Viewer (Carl Zeiss, Jena, Germany) at the magnification of 13×. For each individual focal population assessed, both phenotype expression and proliferative status were noted, BrdU counts being performed by assessing 100 to 600 cells (depending on the lesion size), with an average of approximately 300 cells, and results being expressed as labeling indices (%). The lesions were classified into one of 5 different classes on the basis of expression of the following 5 different enzyme alterations: a) presence of GST-P; (b) presence of G6PD; (c) absence of G6Pase; (d) absence of ATPase; (e) presence of GGT. For example, class 1 lesions demonstrated one out of the above 5 enzyme alterations. Similarly, class 3 lesions had 3 out of the above 5 enzyme alterations in any combination, whereas class 5 lesions were characterized by expression of all 5 enzyme alterations. Respective fractions from class 1 to 5 lesions were expressed as relative numbers (%) of the total lesions included for counting. Four different histopathological categories (274 ACF, 47 HN, 99 HCC and 5 THC) were assessed. The smallest ACF included were 0.2 mm in diameter. The criterion for distinguishing ACF from HN was the presence of obvious compression of the surrounding parenchyma in the latter lesion.

Statistical analyses were performed using the chi-square test to establish the significance of differences in the numbers of lesions divided into various classes, the Mann-Whitney test for the population of lesions in each histopathological category and Student's t test for BrdU labeling indices and size of lesions.

RESULTS

Quantitative analysis of lesions allocated to the different expression classes of altered enzyme phenotypes revealed clear differences depending on the stage of progression. ACF (Fig. 1a) showed a gradient of decrease in numbers of lesions from classes 1 to 5, i.e. class 1, 79 (28.8%); class 2, 68(28.8%); class 3, 58(21.2%); class 4, 58(21.2%); class 5, 11(4.0%). In HN, in contrast, a gradient of increase from classes 1 to 5 was observed. In other words, the lesions exhibiting 4 to 5 enzyme alterations predominated (Fig. 1b). The results were: class 1, 2 (4.3%); class 2, 2(4.3%); class 3, 7(14.9%); class 4, 12
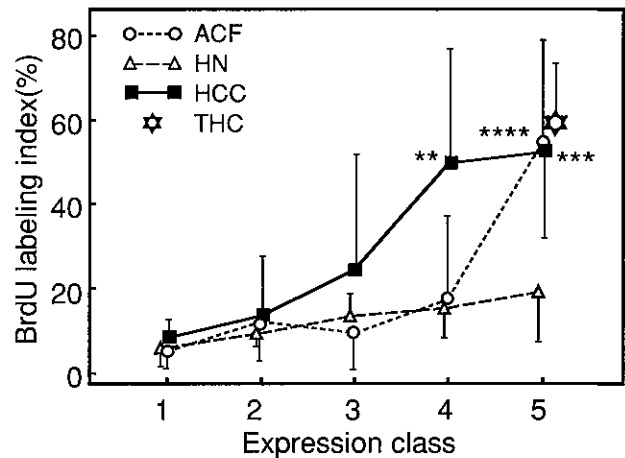

Fig. 2. BrdU labeling indices for ACF, HN HCC and THC. Significantly higher than 4 (∗∗∗∗), 3 (∗∗∗) and 2 (∗∗) lower classes ($P<0.001$–0.05, t test).

(25.5%); class 5, 24(51.1%), a significant increase as compared to ACF ($P<0.001$, Mann-Whitney test). In HCC (Fig. 1c): class 1, 6(6.1%); class 2, 17(17.2%); class 3, 18(18.2%); class 4, 28(28.3%); class 5, 30 (30.3%), a significant increase relative to ACF ($P<0.001$, Mann-Whitney test), but a decrease relative to HN ($P<0.01$, Mann-Whitney test). THC (Fig. 1d) were all in class 5 ($P<0.001$ and $P<0.03$, respectively, Mann-Whitney test as compared to ACF and HCC).

BrdU labeling indices were significantly higher in class 3 to 5 lesions in each histopathological category with the exception of HN (Figs. 2–4). The class 5 ACF demonstrated significantly greater labeling indices than all other classes ($P<0.01$–0.05, t test), and the values for class 4 and 5 lesions in HCC were significantly higher than for class 1 to 3 lesions ($P<0.001$–0.05, t test). The class 5 THC showed the highest BrdU labeling index in the present experiment, but no comparison was possible due to the absence of other class lesions. HN did not show any clear correlation between BrdU labeling index and altered enzyme expression classes.

The diameters of lesions, with data for ACF and HN combined, demonstrated a significant positive correlation with increase in the degree of expression class (Fig. 5) ($P<0.001$–0.05, t test).

DISCUSSION

Quantitative analysis of lesions expressing multiple altered enzyme phenotypes revealed that numbers of simultaneously expressed altered enzyme phenotypes showed a gradient increase in the course of preneoplastic
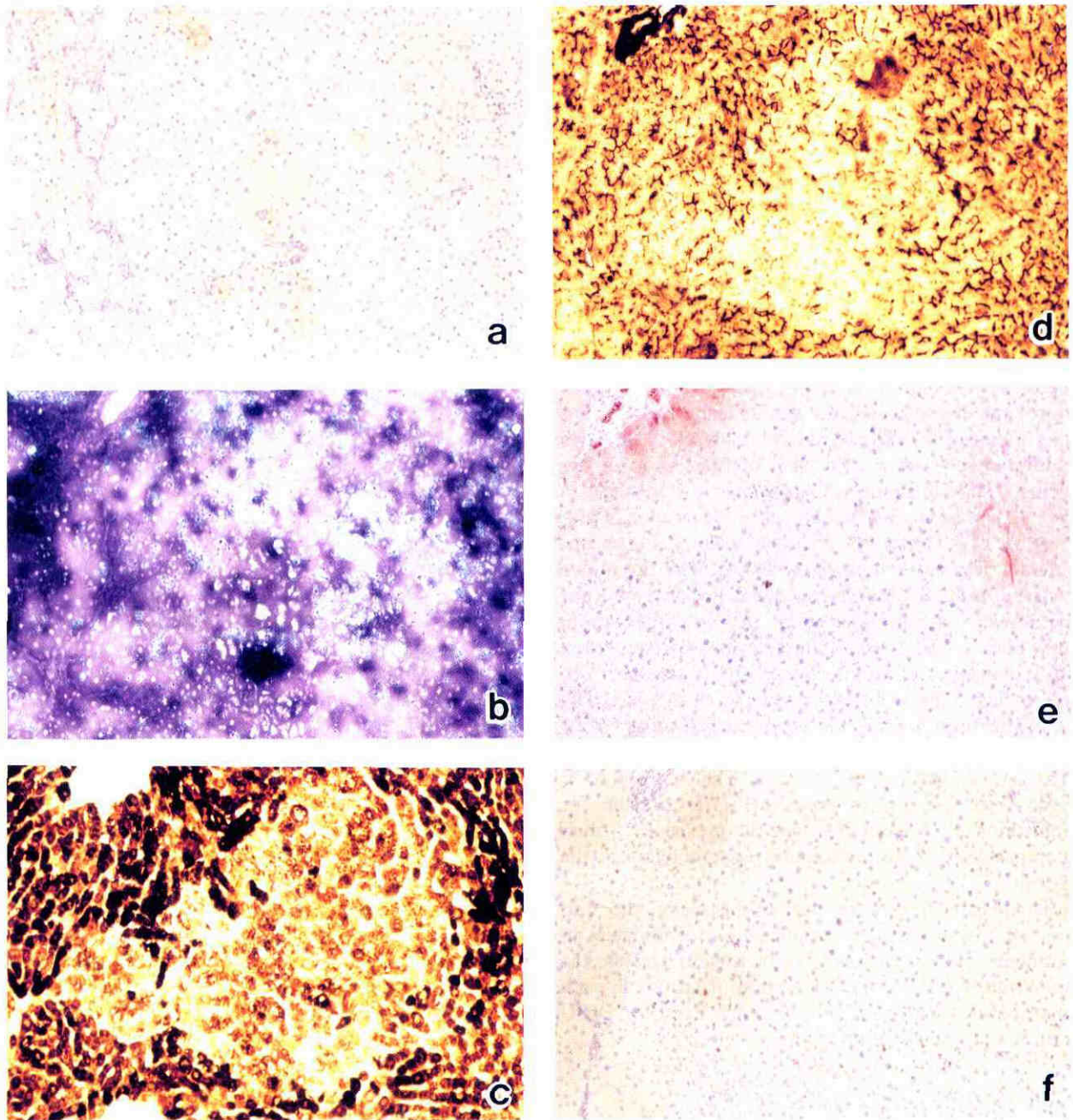
Fig. 3. A class 2 focus in a rat killed at week 20. The lesion almost lacks altered expression of GST-P, G6PD and GGT. Nuclear labeling of BrdU is not markedly different from that in surrounding hepatocytes (×80). a, GST-P; b, G6PD; c, G6Pase; d; ATPase, e; GGT, f, BrdU.

development. In other words, the expression class distribution profile within the same category of lesion showed a clear shift in predominance from low class in ACF to high class in HN. This indicates that increase in the *numbers of simultaneously expressed altered enzyme*

phenotypes is associated with advancement of preneoplastic lesions. Furthermore, progression of preneoplasias to obvious carcinomas such as HCC and THC was closely associated with increase in cell proliferation. *The fact that only ACF in the highest expression class*
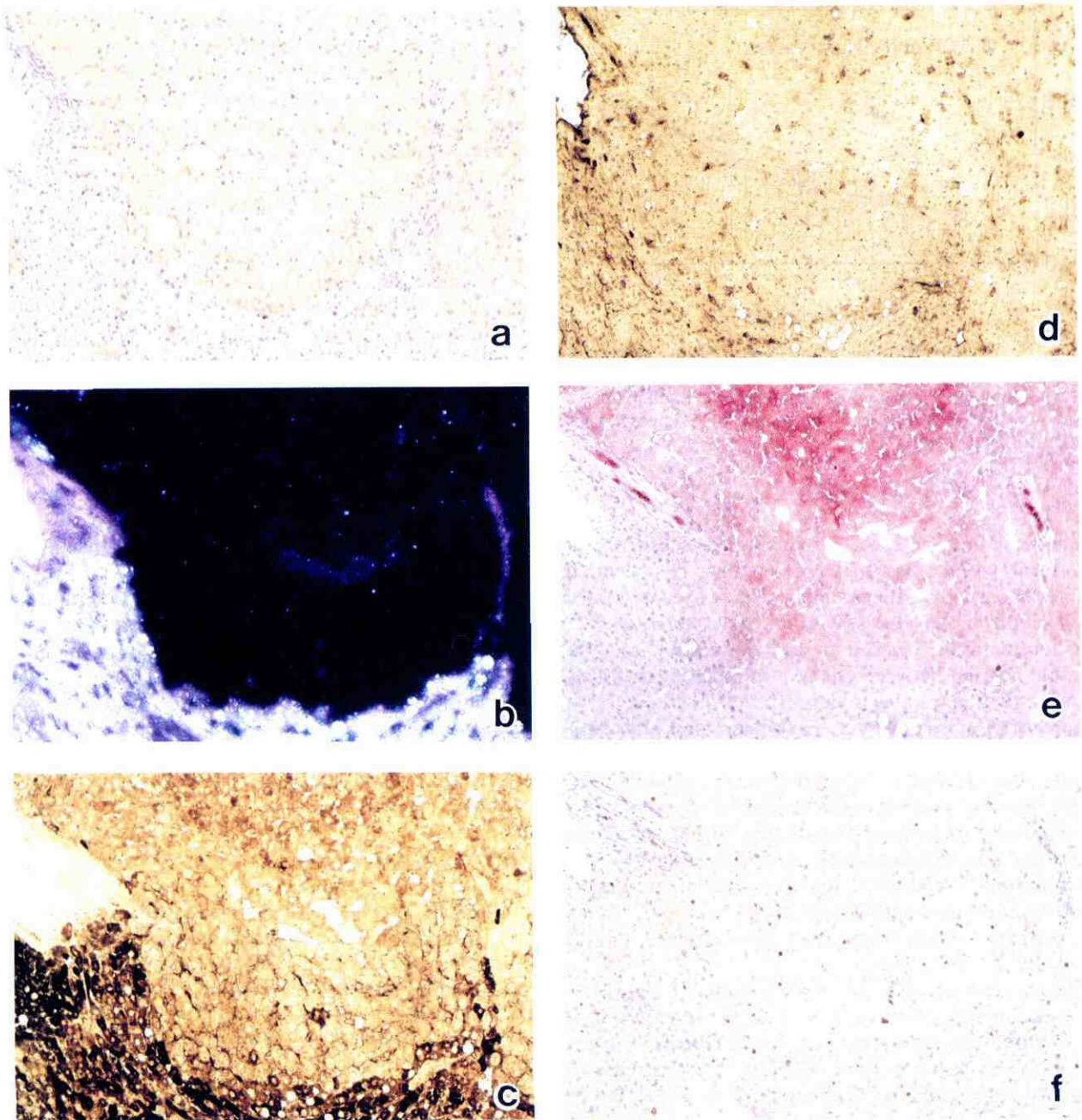
Fig. 4. A class 5 HCC with high BrdU incorporation in a rat killed at week 52 (×50). Note the remarkable difference in the numbers of BrdU-labeled cells. a, GST-P; b, G6PD; c, G6Pase; d; ATPase, e; GGT, f, BrdU.

exhibited a high BrdU labeling index may suggest that these lesions are particular candidates for further neoplastic development.

There have been many reports concerning heterogeneity in phenotypic expression with preneoplastic and neoplastic liver cell lesions.[16-22] However, some authors have

suggested that the enzyme phenotypes of rat liver preneoplastic lesions is not random, but rather that it is the reflection of a directed shift in the biochemistry of the component cells, in some way conferring advantage and therefore of adaptive significance for their own proliferation.[16-19] In addition to changes in expression of drug-
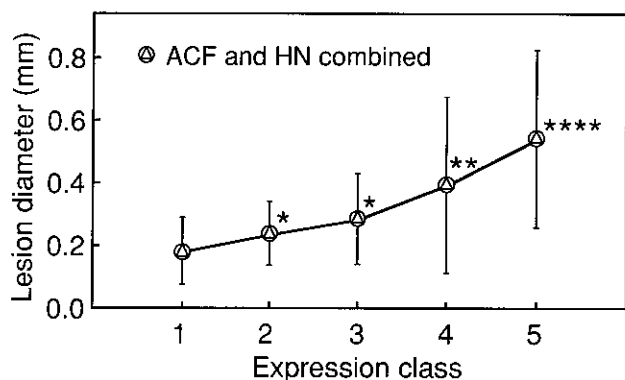
Fig. 5. Correlation between lesion diameter and the numbers of simultaneously expressed altered enzyme phenotypes. ACF and HN were assessed in combination. Significant increases over 4 (✴✴✴✴), 2 (✴✴) and 1 (✴) lower classes ($P < 0.001$–0.05, t test).

metabolizing enzyme species, reducing the sensitivity of such focal populations to toxic agents,[17-19] concerted changes in carbohydrate metabolism have been described which could act to increase proliferative potential.[20-22] This consideration has led to the expectation of considerable overlap between changes in different enzymes, assuming they play roles in concert.[16]

Increase in altered enzyme expression class was especially evident under conditions leading to rapid growth of lesions[23] as observed in the present study. Although not always the case, increase in the number of simultaneously expressed altered enzyme phenotypes within individual lesions has generally been associated with elevated BrdU incorporation,[24] and the present results are in accordance with this tendency. The linear correlation between size expression class found here is of interest in this context.

The general increase in the proportion of high class lesions during the course of preneoplastic development is also essentially in agreement with earlier reports by investigators using fewer enzyme markers.[25-28] Decrease of high class fractions in HCC as compared to HN could be due to genetic instability in advanced stage malignancies, which have already escaped normal growth control, leading to marked phenotypic variation. While the BrdU

labeling indices of HN did not demonstrate any significant variation among altered enzyme expression classes and were found to have lower values than HCC, THC and class 5 ACF, this was presumably due to their increasing size acting to restrain growth in poorly vascularized portions. Intra-lesion variation in growth populations was not taken into account.

In the present study, lesion development was not under any exogenous influence, so the possibility that agents capable of modification had affected the observed enzyme expression[24, 29] could be discounted. In general, neoplastic development is thought to be linked to an increase in simultaneous expression of altered enzyme phenotypes.[30, 31] A number of reports have suggested that this is associated with the emergence of a more homogeneous pattern in the majority of lesions.[32, 33] The fact that peroxisome proliferators such as clofibrate and di(2-ethylhexyl)phthalate can, however, reduce altered enzyme expression must be borne in mind in applying marker enzymes.[34]

In conclusion, the numbers of simultaneously expressed altered enzyme phenotypes shows a clear correlation with preneoplastic development, and proliferative potential is associated with further progression in EHEN-induced rat hepatocarcinogenesis. In this context, it is of particular interest that the THC were, without exception, all class 5. This indicates that higher class lesions have advantages at each hepatocarcinogenic stage which might make them the most important precursors for further neoplastic development and malignant conversion.

ACKNOWLEDGMENTS

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