COMMENTARY

Preneoplastic lesions as end points in carcinogenicity testing.
I. Hepatic preneoplasia

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Introduction

The evaluation of the carcinogenic risk deriving from chemical compounds depends mainly on conventional histopathology up to the present. The accepted end point in carcinogenicity testing is the tumor as defined histologically. A great disadvantage of this approach is the long lag period in the development of tumors induced by chemicals. In order to overcome this drawback, many efforts have been made to detect early biological or morphological lesions which might be specific for carcinogens. A great number of short-term tests carried out in vitro provided valuable information about the reactions of cellular constituents and biochemical macromolecules to the chemicals tested, but they did not allow an unequivocal prediction of the carcinogenic potential of the respective compounds in whole animals, not to mention man. The introduction of modern micromorphological methods such as electron microscopy and cytochemistry in the evaluation of whole-animal studies also revealed many new aspects on carcinogen-induced cellular and subcellular alterations which appeared to be unreliable as indicators of the carcinogenic risk of chemicals. However, during the past two decades a number of characteristic cellular changes has been detected in various tissues, especially in the liver. These changes regularly precede the development of certain tumor types, and are regarded as preneoplastic lesions (1, 2). The altered cell populations usually form well-defined foci. They appear prior to the development of tumors in the target tissue of the carcinogen, and should be duly considered in the evaluation of the carcinogenic risk from chemicals in bioassays.

Definition of preneoplasia

The definition of preneoplasia has to include pregresses of both benign and malignant neoplasms, especially since many observations suggest that benign tumors are often nothing but intermediate stages in the development of malignant neoplasms. Hence, 'preneoplasia' is not identical to 'precancer'. At the histological level, preneoplastic lesions may be defined as phenotypically altered cell populations which have no obvious neoplastic nature, but have a high probability of progressing to a benign or malignant tumor. Theoretically, such a lesion might consist of preneoplastic cells which are only fully transformed after additional intracellular changes (2). We have to admit, however, that other types of focal lesions may also contain neoplastic cells which might be mixed with preneoplastic cells or even occupy the whole lesion. Thus, the definition of preneoplastic lesions used in this commentary refers to the histological level and does not necessarily mean that the lesion is made up of preneoplastic cells. The important question as to whether the conception of preneoplasia is only useful at the organizational level of the tissue or can also be applied to single cells will be discussed later on.

In human and experimental pathology, 'hyperplasia' has often been regarded as an early stage in tumor development (3). However, the use of the term hyperplasia in the context of carcinogenesis entails a dilemma. By definition, hyperplasia is an increase in the number of tissue-specific cells which depends on extracellular growth-stimulation factors, such as hormones, whereas neoplasia is an 'autonomous' increase in the number of cells which is independent of such stimuli. A hitherto unsolved problem is that early stages of neoplastic development may be characterized by a proliferation of cells which cannot be clearly distinguished from normal cells. Although such lesions may have the potential to progress to tumors without any further exposure to known carcinogenic or growth-stimulating factors, they are frequently classified as hyperplasia. It is difficult to avoid this classification as long as we are unable to identify preneoplastic or early neoplastic lesions in many tissues. However, this problematical nomenclature by no means implies that hyperplasia in its strict sense is a prerequisite for neoplasia. At least in chemical carcinogenesis, it is highly probable that proliferating precursor lesions of tumors are already composed of irreversibly altered cells which are not comparable with proliferating normal cells in true hyperplasia, no matter whether we can detect the irreversible cellular changes or not. Because of the uncertainty of the biological behaviour of 'hyperplastic lesions', the discussion on their consideration in the evaluation of carcinogenesis bioassays remains controversial.

Foci of altered hepatocytes

Hepatic preneoplasia has been extensively studied in different species, especially in rats and mice, by many workers using various experimental models (4-9). In rats, preneoplastic hepatic foci have been considered end points of carcinogenicity testing by a number of authors (10-14).

Phenotypic patterns

A landmark for studying early stages of hepatocarcinogenesis was the finding that treatment of rats with nitrosamines produced focal liver lesions which were characterized by an excessive storage of glycogen (15) and usually also by a reduction in the activity of the microsomal enzyme glucose-6-phosphatase (16) as demonstrated by cytochemical methods. From light and electron microscopical investigations of a series of experiments in which rats were treated continuously or over short periods (stop experiments) with N-nitrosomorpholine, a sequence of cellular changes

*Abbreviations: γ-GT, γ-glutamyltranspeptidase; NNM, N-nitrosomorpholine.
has been inferred which leads from clear and acidophilic hepatocytes storing glycogen in excess ('glycogenosis') to basophilic hepatoma cells poor in glycogen, but rich in free or membrane-bound ribosomes (15,17). The preneoplastic clear and acidophilic glycogen storage cells constitute foci which persist after withdrawal of the carcinogen and may progress through intermediate, mixed and basophilic cell foci to neoplastic nodules (adenomas) and hepatocellular carcinomas. This developmental sequence has also been observed during hepatocarcinogenesis induced in rats by a number of other chemicals, such as diethylnitrosamine, dimethylaminoazobenzene or thioacetamide (18), and has been adopted for the classification of specific hepatocellular lesions in rats among which 'foci of altered hepatocytes' were separated from 'neoplastic nodules' by two working groups (19,20). Recently a somewhat more sophisticated nomenclature has been proposed (Table I) which takes into consideration that a certain type of the basophilic foci, called 'tigroid cell focus' (21) apparently differs in many respects from the basophilic foci described earlier (22). Prenecplastic foci which exhibit cytomorphological changes similar to those of the rat have also been detected in other species including primates (7,18,23).

In addition to changes in the amount of the glycogen, the endoplasmic reticulum and the ribosomes, and alterations in the activity of glucose-6-phosphatase, a large number of other cellular changes has been described as 'negative' or 'positive' markers, respectively, for the carcinogen-induced foci (6,9,18,22-29). Examples of enzymes which frequently show a decreased activity such as glucose-6-phosphatase are the membrane-bound adenosine triphosphatase (6,30), acid and alkaline nuclease (31,32), the glycogen phosphorylase (33,34) and adenylate cyclase (35). An increased activity or content has been found in many (albeit not all) foci with 7-glutamyltranspeptidase (7-GT*), glucose-6-phosphate dehydrogenase (34,38), epoxide hydrolase (39,40), uridine-diphosphate-glucuronoyltransferase (41,42), various isoenzymes of cytochrome P-450 (43,44) and glutathione transferases (42,44). Moreover, some other functional alterations have been described in preneoplastic hepatic foci of the rat, namely a resistance to experimental hemosiderosis (45,46), an enhanced glutathione content (47) and a loss of lipid peroxidation (48). The cytochemical changes appearing in focal liver lesions induced with chemicals in mice differ in some respects from those of the rat, but they show also many similarities (7,23).

Observations in different species indicate that the cytochemical pattern of the preneoplastic foci may be rather heterogenous and is apparently influenced by many factors, such as the dose and duration of the carcinogenic treatment, the localization of the focal lesions within the liver lobule and the age, sex and strain of the animals (9,18,26,28,49—51). Some observations suggest that an early reduction of the activity of an enzyme such as glucose-6-phosphatase (52,53) or ATPase (54) might be followed by a reappearance of the enzyme activity or, perhaps, the appearance of an isoenzyme (55) in later stages of hepatocarcinogenesis. Of particular interest is that the activity of γ-GT which has been considered to be a reliable indicator of preneoplastic changes in the liver by a number of laboratories (37) may be partly or totally lacking in certain types of preneoplastic foci in rat liver (21,56—58). On the other hand, a portal focal increase in the activity of this enzyme may also occur in rat liver with age (59) or after partial hepatectomy (60). Two laboratories (61,62) reported that the hepatocarcinogenic peroxisome-proliferating hypolipidemic agent nafenopin may 'suppress' histochemical γ-GT activity in focal hepatic lesions induced by N-2-fluorenylacetamide. In mice, an increased activity of γ-GT has been observed in focal lesions induced by continuous administration of safrole (63) or n-aminoozotoluene (64), but not in those produced by injection of a single dose of diethylthiolsamine in infant animals (23).

In old untreated animals, the various types of foci may develop 'spontaneously', possibly due to a contamination of the food or the environment with small amounts of carcinogens (65,66). An exceptionally high incidence of spontaneous foci in certain rat strains suggests that genetic factors most probably also play an important role (65,66).

### Phenotypic instability

A serious problem for the evaluation of preneoplastic cellular changes in the liver of rodents has become more and more obvious during the last few years. In various experimental models, it was found that most phenotypic 'markers' of hepatocarcinogenesis which have been described so far are not stable and may be mimicked by cellular reactions which are not necessarily related to neoplastic transformation. Thus, under certain experimental conditions, foci (or nodules) have been observed which resemble in their phenotype the persistent lesions discussed, but may disappear after cessation of treatment. In addition to this 'reversion-linked' phenotypic instability, a 'progression-linked' phenotypic instability which is related to metabolic and morphologic changes during transformation of preneoplastic into neoplastic cell populations has to be taken into consideration.

With respect to the reversion-linked phenotypic instability, it has been known for some time that clear cell areas or foci induced by carcinogens may be partly reversible after withdrawal (17,67,68). A partial reversibility of cytomorphological and cytochemical changes, including clear, acidophilic and basophilic features which are similar to those in persistent preneoplastic foci, has also been observed (69) in focal lesions induced in rat liver by the short-term procedure of Solt and Farber (70). A 'reversion', 'remodeling', 'neodifferentiation' or 'maturation' of carcinogen-induced focal liver lesions had earlier been reported by a number of other authors (25,46,71—74). An enhanced cytoplasmic basophilia due to ribosomal increase and accompanied by glycogen reduction as in basophilic hepatic foci may develop in a reversible manner under various pathological conditions, especially after high doses of hepatocarcinogens which frequently lead to so-called megalocytosis (75,76). For an estimation of the extent of phenotypic instability under the experimental conditions of the Solt/Farber system, the recent statement by Farber (77) is of interest: according to his experience, 95—98% of the nodular lesions are reversible, while only 1—3% persist and may progress to hepatocarcinomas. Even if some observations seem to indicate that 'remodeled' lesions may reappear after long lag periods (74,78), the frequently used adjectives 'neoplastic' or 'preneoplastic' for nodules produced in the Solt/Farber system should be avoided. The term 'hepatocyte nodule' proposed by Farber (79) or 'proliferating hepatic nodule' appears to be much

<table>
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<th>Type of focus</th>
<th>Glycogen</th>
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<th>RER/ribosomes</th>
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<td>+</td>
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<td>Acidophilic cell foci</td>
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<td>Mixed cell foci</td>
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more appropriate. The only reliable way to distinguish between the reversible and the persistent focal lesions, the latter being considered as preneoplastic, are stop experiments. Thus, it has been proposed that stop experiments should be conducted whenever foci with a disputed significance develop after application of certain compounds tested (80).

The progression-linked phenotypic instability was already mentioned when the sequential cytomorphological changes during hepatocarcinogenesis were discussed. The results of correlative cytomorphological and cytochemical studies of various enzymes, especially those of carbohydrate and drug metabolism, support this concept. Studies of the key enzymes of the alternative pathways of carbohydrate metabolism suggest that the frequently observed heterogeneous enzyme histochemical patterns do not emerge at random but are characteristic of certain stages in an ordered sequence of metabolic and morphologic changes developing during the process of carcinogenesis (4,34,38). Recent findings of Buchmann and colleagues (44), who investigated the behaviour of a number of enzymes involved in the metabolism of xenobiotic compounds during hepatocarcinogenesis in rats using antibodies to the respective enzymes, indicate that such an ordered pattern of metabolic changes is not only characteristic of the carbohydrate metabolism but also of other metabolic pathways of hepatocytes undergoing neoplastic transformation.

The concept of a progression-linked phenotypic instability is at variance with reports of some other authors who described a phenotypic stability in focal lesions induced by a single dose of the carcinogen in newborn or hepatectomized adult animals followed by phenobarbital treatment (26,81–83). The reason for this discrepancy is not clear at present. An important difference in the evaluation of the experimental results may be that the authors did not determine the histochemical patterns in individual foci but used a statistical approach which did not allow correlation of closely associated changes in metabolic pathways. Another crucial point may be that the additional administration of phenobarbital leads to a rather stable phenotypic expression in the focal lesion, the cause of which is poorly understood at present (84).

**Outset and proliferation kinetics**

The current ideas on the cell cycle-dependent primary interaction of the carcinogen and the possible clonal origin of the preneoplastic cell populations in the liver have been discussed in detail by Rabes (29). Pereira et al. (85) suggested that the initiation of the preneoplastic foci requires formation of O6-methylguanine, but Silinskas et al. (86) have recently shown that formation of O6-methylguanine in rat liver DNA by nitrosamines does not predict initiation of preneoplastic hepatic foci.

Various authors investigated the kinetics of cell proliferation in enzyme-altered foci of the preneoplastic rat liver by autoradiographic determination of the incorporation of [3H]thymidine (29, 30,49,53,87,88). In all of these studies, a considerable increase in cell proliferation was found in the foci as compared with the surrounding tissue or the liver parenchyma of untreated controls. However, most of these studies did not consider the different cell populations composing the foci. When these cell populations were investigated separately it turned out that the incorporation of [3H]thymidine is increased only slightly, if at all, in clear and acidophilic glycogen storage foci (80). A pronounced and steadily increasing cell proliferation was only linked with the appearance of mixed and basophilic cell populations in foci, nodules and carcinomas. These results are in line with the sequence of cellular changes described earlier, but they do not exclude a minor increase in cell proliferation in the clear and acidophilic cell foci. This important aspect would need further clarification, especially since an increase in cell proliferation is a prerequisite for the experimentally well founded hypothesis of a clonal origin of the preneoplastic hepatic foci (89). In contrast to this hypothesis, some observations suggest a simultaneous alteration of many hepatocytes in larger areas of the liver parenchyma (15,17).

**Dose-dependence and relation to neoplasia**

The dose-dependence of the sequential cellular changes induced in rat liver by stop experiments with N-nitrosomorpholine (NNM) has been investigated with morphometric methods (90). The majority of the foci developed after withdrawal of the carcinogen. With all dose levels studied, a sequence was established leading from clear cell and acidophilic cell glycogen storage foci through mixed cell foci and neoplastic nodules to hepatocellular carcinomas. The first appearance and the frequency of the different lesions investigated proved to depend on the dose of carcinogen administered. With increasing dose of NNM, the number of focal lesions considerably increased, and this was accompanied by an earlier development of mixed and basophilic cell populations. There was no indication of any reversibility of the focal lesions under these experimental conditions. On the contrary, the foci became larger and acquired phenotypic markers closer to neoplasia without further action of the carcinogen. A progressive development has also been revealed by morphometric methods for basophilic foci induced with a single injection of diethylnitrosamine in infant mice (91,92).

Histological transitions between focal hepatic lesions, hepatic adenomas (neoplastic nodules) and carcinomas have been described by many authors (17,25,27). While these observations indicate that the adenomas originate from the foci and may progress to carcinomas, it is highly probable that the latter can also develop directly from the foci without going through a nodular intermediate stage (93,94).

In addition to these cytomorphological and morphometric results, alterations in a number of functional cellular changes strongly support the concept of a close relation of foci, nodules and carcinomas. Thus, a similar decrease or increase in the activity of many enzymes has been demonstrated by enzyme histochemical methods in these lesions (18,24—27). Several authors have demonstrated in rats or mice that preneoplastic foci, neoplastic nodules and carcinomas share a common defect in that they do not accumulate iron in a siderotic liver produced artificially (45, 46,95).

Of particular interest are data concerning the dose-dependence and development of enzyme-altered foci as published by several groups (24,96). Although the methods used were somewhat varied, all the results indicate that quantitative correlations exist between the size and the number of foci and the dose and duration of carcinogen treatment. From a statistical point of view there seems to be a good correlation of dose—time dependence for the early foci and for the later development of liver tumors. Although it has not been taken into consideration so far that a considerable phenotypic instability of enzyme-altered foci and nodules induced by hepatocarcinogens has been observed under certain experimental conditions as mentioned earlier (5,69,71,79), most of these findings are in accord with the concept of a precursor–product relationship between the foci of altered hepatocytes and hepatic tumors. In this context, it should be mentioned, however, that a direct relationship between focal liver lesions, nodules and carcinomas has been questioned by some authors (99). Large discrepancies between the number of foci appearing early during hepatocarcinogenesis and final tumor yield are in-
Hepatic foci have been proposed. The most convincing models compound.

ed some type of focal hepatic lesion prior to the development period (11,80) and the animals are investigated up to several weeks or months after withdrawal (stop experiment). In an extreme type of stop experiment a single dose is applied to newborn animals (92,102) or to adult animals after partial hepatectomy (100,103). Frequently, the additional application of phenobarbital has been used to ‘promote’ the ‘initiated’ cells possibly induced by the test compound, either in young animals (98,104) or in adult animals after partial hepatectomy (26,105). This approach has been adopted for the so-called ‘rat liver foci bioassay’ (10, 14). Although the additional application of phenobarbital renders this system more ‘sensitive’, many problematical interpretations are based on this ‘two-stage model’ since most authors imply that the operational distinction between the ‘initiator’ and the ‘promoter’ allows mechanistic explanations. However, a controversial discussion is still going on as to whether phenobarbital merely promotes initiated cells or acts in accord with the ‘initiator’ as a weak carcinogen (81,106-108). Nevertheless, the ‘rat liver foci bioassay’ has been widely used during the past few years (10-14).

In a recent review, Pereira and Stoner (14) compared the rat liver foci assay with the strain A mouse lung tumor assay to detect carcinogens. The authors stated that the rat liver foci assay was sensitive to 69% of 54 compounds found to be carcinogenic in long-term bioassays and the strain A lung tumor assay to 54% of 93 carcinogens. None of the 10 compounds found to be non-carcinogenic in long-term bioassays were active in the rat liver foci assay, while seven of 23 non-carcinogens (30%) were active in the lung tumor assay. Ten of the 17 carcinogens negative in the rat liver foci assay are believed to exhibit tumor-promoting activity, three are direct acting alkylating agents (dimethylsulfate, epichlorohydrin and β-propiolactone), and the remaining three are azobenzene, 1,2-dibromoethane and thiaoacetamide. Thirty-two of the 43 carcinogens negative in the lung tumor assay were active in either (i) the mouse liver only, (ii) the rat and not in the mouse or (iii) in both the rat and mouse liver but not in other organs of the mouse.

Extremely complex models have been developed on the basis of an experiment described by Teebor and Becker (109) and additional considerations derived from other studies (70,110-112). From a review taking into consideration 80 compounds tested, Parodi et al. (113) concluded that the ‘preneoplastic nodules’ induced by these complex procedures were more predictive than the Ames test as a statistical trend. However, there was a somewhat better predictivity of the ‘preneoplastic nodules’ for liver tumors, as compared with other tumors. It has been emphasized earlier that all of the complex models mentioned should be interpreted very cautiously (80). The reversibility of many foci and nodules appearing in these models after cessation of treatment does not seem to be a major problem from a practical point of view since some foci and nodules persist in all models described. Therefore, any production of foci and nodules might be sufficient to indicate carcinogenic effect on the liver. However, the additional application of chemicals, some of which are potent carcinogens in these test systems, hampers the interpretation of the results as long as we know little about the different effects of the various experimental procedures. In any case, stop experiments are to be recommended in order to discriminate clearly between reversible and persistent foci and nodules, the persisting lesions being regarded as preneoplastic (foci) or neoplastic (nodules) in nature (80).

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