Advantages and Limitations of Stereological Estimation of Placental Glutathione S-Transferase-positive Rat Liver Cell Foci by Computerized Three-dimensional Reconstruction

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The applicability to a medium-term bioassay for liver carcinogens of mathematical formulae for the calculation of numbers of foci per volume was examined in F344 rats. Two weeks after initiation with diethylnitrosamine, animals were given test compounds for 6 weeks, partial hepatectomy being performed at week 3. At week 8, the rats were killed, the livers removed and stained immuno-histochemically for assessment of glutathione S-transferase P form (GST-P)-positive foci development. Numbers and areas of lesions were measured two-dimensionally using a color image analyzer, and the Enzmann and Campbell formulae for estimation of number and volume per cm^3 were applied to the results. In addition, three-dimensional reconstruction of individual foci was performed using up to 150 GST-P stained foci, with the aid of a computerized graphic system. Both two- and three-dimensionally expressed quantitative results were found to adequately demonstrate the modifying potential of test chemicals on hepatocarcinogenesis. The three-dimensional approach was only more accurate if most of the foci were small and the liver was enlarged by compound treatment. Stereological reconstruction revealed that the shape of GST-P-positive foci, especially if relatively large, is not always spherical but that many demonstrate irregular branching forms, so that the assumptions behind stereological estimation are not met. The results therefore show that care must be taken in applying mathematical formulae for the calculation of three-dimensional data.

Key words: Rat liver — Stereology — Enzyme-altered foci — Glutathione S-transferase — Preneoplastic lesion

Quantitative analyses of enzyme-altered hepatocyte foci in the rodent liver have often been used to assess modification of carcinogenesis, including promoting or inhibiting activities of test chemicals.[1-3] Measurement of the number and the area of foci per cm^2 with the aid of an image analyzer has been applied in some laboratories,[4, 5] but this two-dimensional (2-D) evaluation has the disadvantage of underestimating the yield of small lesions which have a lower probability of being transected. Therefore three-dimensional (3-D) values have been recommended and a number of different stereological formulae have been designed for estimation of the number and the volume of foci per cm^3, taking into account the size distribution of lesions measured in two-dimensions.[6-12] Thus mathematics has been applied to extrapolation from 2-D number and area data to give 3-D values. However, these stereological formulae are based on critical assumptions, i.e. that all foci are independent and also spherical.

The present report is concerned with whether these assumptions are indeed correct. With this aim, the relationship between rat liver preneoplastic foci values gained from 2-D measurement and 3-D estimation by application of formulae was investigated in animals treated with weak and strong hepatocarcinogens, including chemicals which markedly enlarge the liver volume such as polychlorinated biphenyls (PCB). In addition, the 3-D shapes of individual enzyme-altered foci were reconstructed using a 3-D computerized graphic system.

MATERIALS AND METHODS

Chemicals The chemicals used were obtained from the following commercial sources: diethylnitrosamine (DEN, Tokyo Chemical Industry Co., Ltd., Tokyo); N-ethyl-N-hydroxyethylnitrosamine (EHEN, Izumi Chemical Co., Osaka); dibutylnitrosamine (DBN, Tokyo Kasei Co., Tokyo); phenobarbital (PB, Iwaki Seiyaku Co., Tokyo); aldrin (Nakarai Kagaku Co., Tokyo); butylated hydroxyanisole (BHA, Wako Pure Chemical Industry Ltd., Osaka); PCB (Kanegafuchi Chemical Co., Osaka); N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN, Izumi Chemical Co., Yokohama).

Induction of enzyme-altered foci in the liver A total of 120 male Fischer rats (Charles River Japan Inc., Atsugi) weighing about 160 g were maintained on basal diet (Oriental M, Oriental Yeast Co., Tokyo) *ad libitum* and housed in plastic cages in an air-conditioned room at 24±2°C. The animals were given a single ip injection of DEN at a dose of 200 mg/kg body weight dissolved in

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0.9% NaCl to initiate hepatocarcinogenesis. After 2 weeks on basal diet, they received one of the following compounds in the basal diet (D) or in drinking water (W): EHEN, 0.05% W; DBN, 0.1% W; PB, 0.05% D; aldrin, 0.005% D; BHA, 2% D; PCB, 0.1% D; BBN, 0.2% W or basal diet alone. All animals were subjected to partial hepatectomy (PH) at week 3, and sacrificed at week 8, when the livers were excised and cut with a razor blade into 2–3 mm thick slices. Tissues were fixed in phosphate-buffered formalin or ice-cold acetone for subsequent immunohistochemical demonstration of glutathione S-transferase P form (GST-P)-positive foci.

Immunohistochemical staining and quantitative analysis
GST-P staining was performed as described previously[13, 14] using the avidin-biotin-peroxidase complex (ABC) method.[15] Paraffin sections were routinely passed through petroleum benzene and a graded alcohol series and then treated sequentially with normal goat serum, rabbit anti-GST-P (1:8000), biotin-labeled goat anti-rabbit IgG (1:400) and ABC. The sites of peroxidase binding were demonstrated by the 3,3′-diaminobenzidine method. Sections were then counter-stained with hematoxylin for microscopic examination. As a negative control for the specificity of anti-GST-P antibody binding, pre-immune rabbit serum was used instead of antiserum.

The numbers and areas of GST-P-positive foci more than 0.2 mm in diameter were measured using a color video image processor (Spicca Computer System, Nippon Avionics Co., Ltd., Tokyo). Total section area of the liver was also measured, and the numbers and areas of foci per unit area (cm²) were calculated.

The 3-D quantitative values for GST-P-positive foci were calculated by applying Campbell et al.'s[6] and Enzmann et al.'s[16] formulae. Furthermore, the numbers

of foci per liver were also calculated based on measured liver weights (the specific gravity of liver was assumed to be 1).

For 3-D reconstruction of the foci of the liver, paraffin-embedded liver was serially sectioned (5 μm thick) and immunohistochemically stained with GST-P at 10 to 15 μm steps (every 2 or 3 sections) for up to 150 sections. Several GST-P-positive foci and nodules were selected and reconstructed 3-dimensionally with the aid of a computer graphics system (Spicca Computer System-TRI, Nippon Avionics Co., Ltd.).

Statistical analysis was carried out using Student's t test.

Table I. Numbers and Areas of Two-dimensional GST-P-positive Foci in the Livers of Rats Receiving Different Treatments

Chemical	No. of rats	Liver weight (g)	GST-P-positive foci	
			No./cm²	Area(mm²)/cm²
EHEN	14	8.0±0.5[a]	129.7±13.7[a]	24.19±4.23[a]
DBN	14	6.4±0.4	56.2±17.3[a]	5.08±2.94[a]
PB	15	9.4±0.8[a]	21.4±9.0[a]	4.06±3.13[a]
Aldrin	14	8.9±0.6[a]	17.8±6.4[a]	1.50±0.98[b]
BHA	14	9.0±0.6[a]	2.5±0.8[a]	0.16±0.05[a]
PCB	12	12.0±0.7[a]	24.8±7.6[a]	2.50±1.05[a]
BBN	14	7.1±0.3[a]	6.8±2.1	0.60±0.30
Control	14	6.6±0.3	6.4±1.9	0.47±0.20

a) Statistically different from the control group value at $P<0.001$.
b) Statistically different from the control group value at $P<0.01$.

Table II. Three-dimensional Calculated Results for Numbers and Volumes of GST-P-positive Rat Liver Foci Using the Enzmann and Campbell Formulae

Chemical	No. of rats	GST-P-positive foci							
		Enzmann et al.'s formula				Campbell et al.'s formula			
		No./cm³	No./liver	Vol.(mm³)/cm³	Vol./liver	No./cm³	No./liver	Vol.(mm³)/cm³	Vol./liver
EHEN	14	3903±565[a]	31719±6593[a]	23.2±4.0[a]	188.4±40.1[a]	3828±628[a]	31161±7033[a]	43.1±7.6[a]	350.3±77.3[a]
DBN	14	2301±678[a]	14721±4858[a]	4.9±2.9[a]	31.6±19.6[a]	2251±796[a]	14354±5274[a]	9.4±5.4[a]	60.3±37.5[a]
PB	15	727±364[a]	6885±3710[a]	3.9±3.1[a]	36.3±28.2[a]	638±310[a]	6044±3184[a]	7.8±5.8[a]	72.5±52.3[a]
Aldrin	14	820±333[a]	7241±2757[a]	1.3±0.9[b]	11.5±7.2[a]	813±314[a]	7194±2641[a]	2.9±2.1[b]	25.2±16.9[b]
BHA	14	130±40[a]	1172±364[a]	0.2±0.1[a]	1.7±0.5[b]	118±36[a]	1062±322[b]	0.3±0.1[a]	3.0±0.9[a]
PCB	12	990±390[a]	10765±4474[a]	2.5±1.1[a]	30.5±12.6[a]	1232±828[a]	14787±9938[a]	4.9±1.9[a]	58.3±23.1[a]
BBN	14	312±91	2207±660	0.5±0.2	3.4±1.4	280±98	1979±686	0.1±0.5	7.0±2.9
Control	14	291±85	1909±556	0.5±0.2	3.0±1.2	262±91	1716±586	0.9±0.4	6.0±2.4

a) Statistically different from the respective control group value at $P<0.001$.
b) Statistically different from the respective control group value at $P<0.01$.

RESULTS

Quantitative data for GST-P-positive foci per cm^2, and calculated numbers of foci per cm^3 and per liver for each of the compounds investigated are summarized in Tables I and II. The liver weights of rats treated with PCB were twice the control values, and increases were also observed with the PB and BHA treatments. The promoting potentials of EHEN, DBN, aldrin, PCB and PB or the inhibition by BHA are equally well expressed as either numbers of foci per cm^2 or as calculated numbers per cm^3,
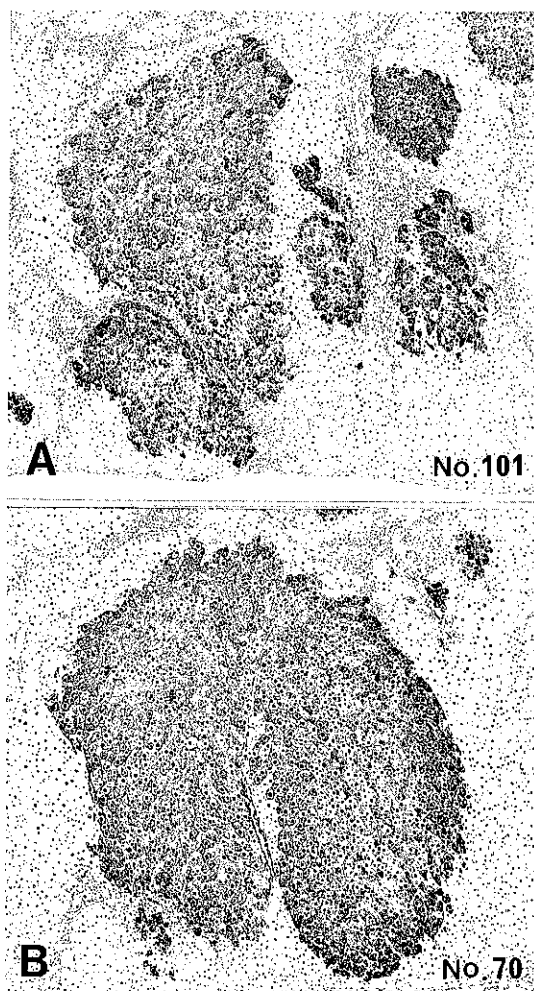

Fig. 1. Two-dimensional sections of GST-P-positive liver cell foci of rats treated with DEN→EHEN+PH. Two sample sections from a series, A) section No. 101 and B) No. 70, illustrate a single focus confirmed by 3-D reconstruction (see Fig. 2). A) Separate adjacent foci are apparent. B) Only one focus is apparent.

irrespective of the formula employed. Although the liver enlargement caused by PCB treatment did not affect the 2-D value statistically, the value did not reflect the actual number and area of foci. The 3-D value better reflected the actual values. Both 2-D and 3-D yielded quantitative results demonstrating the same modifying potential for all the chemicals tested in the medium-term bioassay.

Figure 1 illustrates the 2-D appearance of a GST-P-positive liver cell focus in a rat treated with DEN→ EHEN+PH. Two sample levels through the lesion, confirmed to be a single entity, are shown, and although only one focus is evident in Fig. 1-B (No. 70), several separate adjacent foci are apparent in Fig. 1-A (No. 101). The 3-D reconstructed appearances of large and small GST-P-positive liver cell foci from rats treated with DEN→ PH+EHEN and DEN→PH, respectively, are shown in Fig. 2-A and 2-B. The No. 70 and No. 101 levels in Fig. 2-A correspond to the positions marked in Fig. 1-B and 1-A, respectively. Whereas the small foci induced by DEN→PH were smooth and spherical in shape without branching, some of the larger foci or nodules, observed after DEN→PH+EHEN treatment for example, were very irregular.

DISCUSSION

It is now generally accepted that quantitative data for preneoplastic foci can be used to assess indices of initiating or promoting potential in hepatocarcinogenesis,[17] but some authors have strongly recommended that estimation of effects should be based on 3-D calculated values (No. per cm^3) and not directly measured 2-D values (No. per cm^2), because of inaccuracies with the second approach.[11] However, the results from the present experiments regarding the effects of strong and weak hepatocarcinogens, carcinogens with targets other than the liver, and inhibitors of hepatocarcinogenesis demonstrated clearly that both 2-D and 3-D values calculated using 2 different formulae give essentially the same answers with similar estimation of degree. Thus, it can be concluded that 2-D values are adequate to indicate the modifying potential of the chemicals in the investigated medium-term bioassay for liver carcinogens and promoters.

Furthermore, the mathematical transposition to 3-D numbers and volumes of liver cell foci can itself introduce inaccuracies, since all the available formulae are based on the assumption of spherical lesions. Application of the 3-D reconstruction methodology which has been developed from investigations in a wide variety of fields[18-20] unequivocally demonstrated, however, that this is not the case. Rather it should be borne in mind that larger lesions can present as very irregular branching structures. Thus, the reconstruction technique proved of
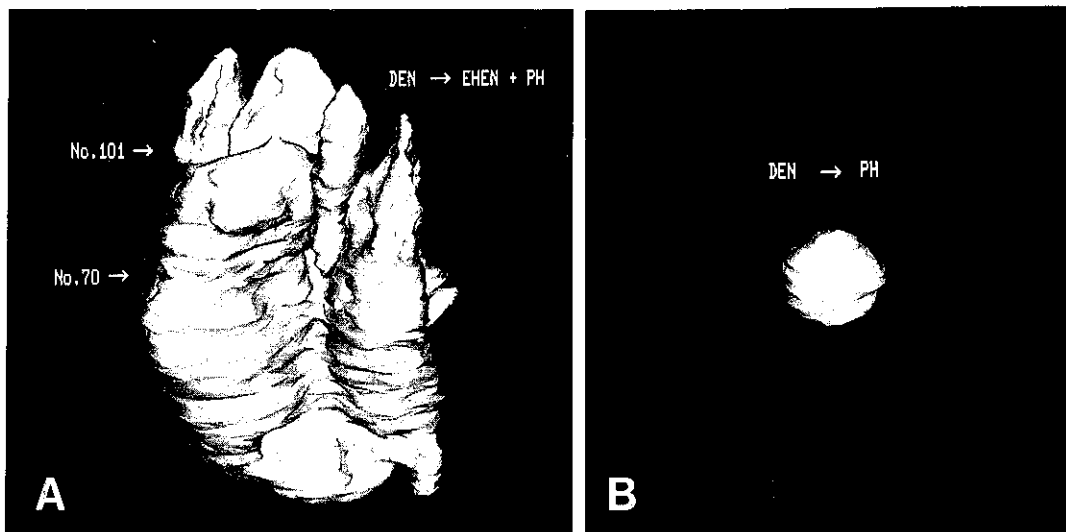
Fig. 2. Three-dimensional reconstructed appearances of A) large and B) small GST-P-positive liver cell foci in rats treated with DEN→EHEN + PH and DEN→PH, respectively. The No. 101 and No. 70 levels illustrated in Fig. 2-A correspond to the positions illustrated in Fig. 1-A and 1-B, respectively. Note that the focus is not spherical and has several branches at the level of No. 101 in Fig. 2-A. Fig. 2-B shows that the small focus is spherical with no branching.

great value as shown earlier for analyses of liver cirrhosis[21, 22] and gastric tumors.[23, 24]

In addition to the criticism that lesion number might be overestimated and size correspondingly decreased by double counting of branching foci and nodules, a second point regarding transposition deserves mention. Comparison of the results using the formula of Campbell et al.[6] with those (which were almost twice as large) using the Enzmann et al.[16] method clearly shows a striking variation. Thus, a multiplying factor regarding measurement inaccuracies can be introduced which largely depends on the formula applied. Further studies appear necessary

before application of stereological estimation to liver cell foci can be unreservedly recommended.

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