ACTA STEREOL 1985; 4/2: 225—229
PROC ESS IV-1 GÖTEBORG 1985
ORIGINAL SCIENTIFIC PAPER

AUTOMATED KARYOMETRY OF HEPATOCYTES DURING EXPERIMENTAL LIVER CARCINOGENESIS IN NORMALLY - FED AND PROTEIN - DEFICIENT RATS

Fatima Zora El Kebir*, Marie-Thérèse Chalumeau** and Jean Paul Rigaut***

* Institut de Biologie, Université d'Oran, Es Senia, Oran, Algeria.

** Laboratoire de Biologie du Développement, U.E.R. Biomédicale, 74 rue Marcel Cachin, 93012-Bobigny-Cédex, France.

*** Unité de Recherches Biomathématiques et Biostatistiques (U.263 -INSERM), Université Paris 7, 2 Place Jussieu, 75251-Paris Cédex 05, France.

ABSTRACT

Karyometric results, with stereological sphere unfoldings, from rat liver hepatocytic nuclei, have been obtained after an experimental carcinogenesis (Farber) protocol. The rats were protein deficient, or 20% protein fed. A special image analysis algorithm for the IBAS (Kontron) has been used. The non-parametric unfolding method uses profile area classes. Some difficulties were encountered in the interpretation of the karyometric results. The positioning of the diploid and tetraploid peaks changes in normal animals with age. Some guesses can nevertheless be made at ploidy values. Larger nuclei are observed at 4 months after the onset of the protocol in protein-deficient treated animals. At 5 and 7 months, practically only diploid-sized nuclei are found in treated animals. The results are consistent with a possible early polyploidization during oncogenesis, followed by a re-diploidization. The difficulties in interpreting some histograms show that more studies should be done, and notably comparisons with DNA histograms on smears or by cytoflow.

Keywords: Experimental cancer, image analysis, karyometry, liver, protein deficiency, sphere unfolding.

INTRODUCTION

Increased nuclear DNA contents have been reported in various human and experimental lesions considered as associated with an increased risk of subsequent cancer (review in Rigaut et al., 1985), including those observed during experimental liver carcinogenesis, starting in the very first stages (Stich, 1960; Christie and Le Page, 1961; Romagna and Zbinden, 1981), although in that case contradictory results have been reported (Schwarze et al., 1984). As a well-documented correlation exists between nuclear size and DNA content (review in Rigaut et al., 1982), a few karyometric studies have been made in experimental pathology, but usually without any stereological estimation when using sections (review in Rigaut et al., 1985).

METHODS

The complete results of a study started in 1982 (preliminary results in El Kebir et al. (1983) and Rigaut et al. (1984)) on liver carcinogenesis by Farber's protocol (Farber, 1980) (diethylnitrosamine/ hepatectomy/ 2-acetylaminofluorene) have been obtained.

The 153 Wistar rats were divided into 2 groups (controls, treated) and subdivided into 10% protein-fed (protein deficient) and 20% protein-fed animals. The protocol was started in 2 month-old animals and approximately equal-sized groups were sacrificed at 1, 4, 5, 7 and 8 months after the onset of the protocol.

A careful standardization of all histological procedures was respected, and 4 µm-thick Feulgen-stained sections were studied with an automated image analysis algorithm for the IBAS. In treated animals, hyperplastic nodules only were studied. They were always present and easily located by the operator before positioning the scanning stage to the starting coordinates.

The most recent, fully automated program includes meander scanning, autofocus and elimination of endothelial nuclear profiles (only the hepatocytic nuclei are studied) by comparisons of grey-level-thresholded images, transformed or not by modified median filtering (Fig. 1). The processing speed of the algorithm is 0.5 nuclear profile per second. The total time required for reading a slide (300-500 nuclear profiles), all inputs included, is approximately 12-15 minutes.

The data (nuclear profile areas from spherical nuclei) are treated by a separate program using, for the sphere size distribution unfolding, a matrix method for profile area classes (Rigaut, 1984), which might be preferable to those using diameters calculated from the image-analyser-measured nuclear profile areas. The model accounts for section thickness and truncation effects.

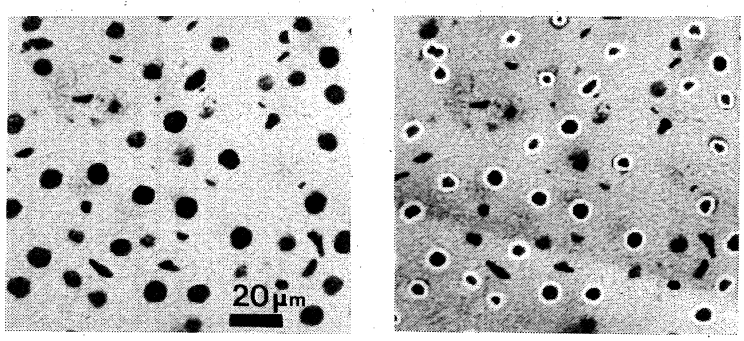

Fig. 1. Images on the IBAS TV monitor: (a) original image of a Feulgen-stained liver section; (b) the white contours show the superimposition of the contours of the binarized nuclear profiles on (a). Note the satisfactory elimination of endothelial cell nuclei. A guard frame is then used to decide which profiles are measured.

RESULTS

The results are more difficult to interpret than when using DNA measurements, as the peaks of same ploidy are not always exactly positioned at the same size value and the pooling of data from different cases in the same group often leads to a filling of some of the spaces between neighbouring peaks. However, some conclusions can be drawn and some guesses may be made at ploidy values.

In all the controls is noted the well-known increase in tetraploid (4n) nuclei with age, but this evolution is faster in protein-deficient rats in the first 4 months. It must be noted that, in the controls, the positioning of the 2n and 4n peaks shifts to smaller values with age.

In the treated groups, as compared to controls, a bigger proportion of large nuclei is observed at 4 months after the onset of the protocol. What is most striking (Fig. 2) is the positioning of the two major peaks, in the treated, at 4 months, at values respectively higher than those which seem to correspond to 2n and 4n ploidies in the controls, rendering difficult any interpretation in terms of ploidies. Larger nuclei are observed at 4 months when compared to 1 month in treated, protein-deficient animals, but not in the others. At 5 and 7 months, practically only small (probably 2n) nuclei are observed in treated animals. At 8 months, larger nuclei often reappear in treated animals; many cancers start at that time, and their ploidy value is known to be quite variable, although sub-tetraploid values seem the most common (Stich, 1960).

It must be noted that the ratios between the positions of all major peaks point to modal (2:1) volume ratios (sphere equatorial area class ratios to the power 3/2).

On the whole, difficulties in interpretation make it impossible to guess what precise ploidy values are present in treated animals at 1 and 4 months after the start of the Farber protocol. It is possible, however, to conclude that (i) an important proportion of the nuclei are larger than in controls at 4 months in the treated, protein-deficient animals, but not in the treated, 20% protein-fed ones; (ii) at 5 and 7 months, in both treated groups, only small-sized (probably diploid) nuclei are observed; and (iii) larger nuclei often reappear in treated animals at 8 months, an age at which many hyperplastic nodules are usually regarded as being cancerous.

DISCUSSION

It is clear, by the difficulties encountered in the interpretation of the ploidy values of the size distribution peaks, that a correlation with DNA values is necessary, and this is being done now. Some preparative and instrumental errors cannot be excluded, although they seem to have been kept minimal. When both nuclei from a binucleated cell produce a profile, those sometimes overlap and cannot be separated by the image analyser, which then eliminates them (by a form factor evaluation). It could be possible to improve this, and even to evaluate the proportion of binucleated cells by a mathematical model (Rigaut, 1985). The karyometric results, however, were obtained very fast, with an efficient image analysis algorithm, and peaks, after unfolding, are well-defined. The very existence of complexities in the positioning of the peaks is interesting and comparisons with DNA measurements should be rewarding.

Our study has demonstrated an evolution to probable diploid values, through some steps which are difficult to assess (nuclear swelling? hyper-modal ploidism?).

A pattern of polyploidization followed by re-diploidization is suggested in human studies (Rigaut et al., 1982, 1985) on dysplasias. If a comparable evolution could be demonstrated experimentally, this could shed some light on carcinogenetic mechanisms. An increase in both 2n and 8n ploidies (Romagna and Zbinden, 1981) and correlation between enlarged nuclei and increased DNA values (Christie and Le Page, 1961) have been noted during rat liver carcinogenesis. On the contrary, it seems to some authors that the main fact when using a Farber protocol is the fast emergence of 2n nuclei (Schwarze et al., 1984). The explanation of such discrepancies in the literature is not obvious. Some of them could be due to the studied

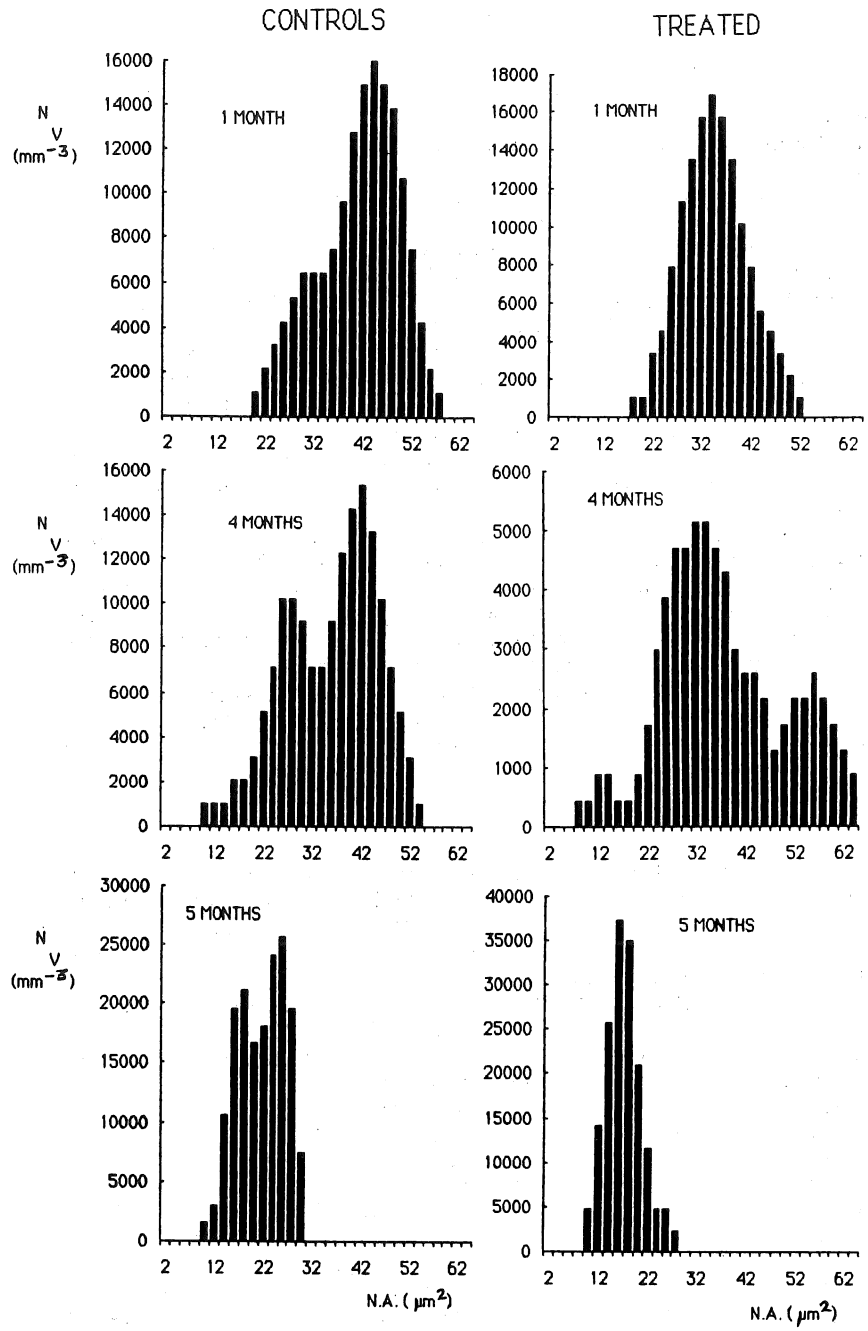

Fig. 2. Some of the unfolded nuclear size distributions (N.A. = nuclear area; sphere equatorial area classes). Protein deficient rats: controls and treated; sacrificed 1, 4 and 5 months after the onset of the Farber protocol.

cellular type and to the age of the animals at the onset of the protocol and at sacrifice. We had previously reported that "oval" (transitional) cells were always diploid during our protocol (El Kebir et al., 1983). Our animals are older at the start than those of Schwarze et al. (1984), which have only 2n cells; they already have normally a majority of 4n cells. Lastly, the differences noted between protein-deficient and normally-fed rats are difficult to explain.

REFERENCES

Christie GS, Le Page R.N. Enlargement of liver cell nuclei: effect of dimethylnitrosamine on size and desoxyribosenucleic acid content. Lab Invest 1961; 10 : 729-743.

El Kebir FZ, Chalumeau MT, Reith A, Rigaut J.P. Stereological unfoldings of nuclear size distributions on liver sections during experimenta carcinogenesis in normally fed and protein-deficient rats. Acta Stereol 1983; 2-S1: 285-288.

Farber E. The sequential analysis of liver cancer induction. Biochim Biophys Acta 1980; 605: 149-169.

Rigaut JP. Non-parametric estimation of sphere size distributions from profile area distributions in sections showing overprojection and truncation. Micron-Microsc Acta 1984; 15: 1-6.

Rigaut JP. The 'bi-corpuscle' stereological problem. Estimation of distributions of distances between paired spheres, from measurements in slabs showing overprojection and truncation, and application to binucleated cells. J Microsc 1985; 138: 189-201.

Rigaut JP, Boysen M, Reith A. Karyometry of pseudostratified metaplastic and dysplastic nasal epithelium by morphometry and stereology .2. automated image analysis (IBAS) of the basal layer of nickel workers. Path Res Pract 1985; 180: 151-160.

Rigaut JP, Margules S, Boysen M, Chalumeau MT, Reith A. Karyometry of pseudostratified, metaplastic and dysplastic nasal epithelium by morphometry and stereology 1. A general model for automated image analysis of epithelia. Path Res Pract 1982; 174: 342-356.

Rigaut JP, Reith A, El Kebir FZ. Karyometry by automated image analysis. Application to precancerous lesions. Path Res Pract 1984; 179: 216-219.

Romagna F, Zbinden G. Distribution of nuclear size and DNA content in serial liver biopsies of rats treated with N-nitrosomorpholine, phenobarbital and butylated hydroxytoluene. Exp Cell Biol 1981; 49: 294-305.

Schwarze PE, Pettersen EO, Shoaib MC, Seglen P.O. Emergence of a population of small, diploid hepatocytes during hepatocarcinogenesis. Carcinogenesis 1984; 5: 1267-1275.

Stich HF. The DNA content of tumor cells. II. Alterations during the formation of hepatomas in rats. J Nat Cancer Inst 1960; 24: 1283-1296.