CYTOPHOTOMETRIC AND AUTORADIOGRAPHIC EVALUATION OF CELL KINETICS IN DECIDUAL GROWTH IN RATS

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Summary. Because of endopolyploidy, cell kinetics in decidual growth are best studied by cytophotometric methods. This technique was applied to smears of nuclei isolated from decidualizing endometrial stroma at various moments after uterine scratching in hormonally prepared rats. After a 48-hr lag period, the production of polyploid nuclei was accelerated. At its maximal development (96 hr), the decidual tissue contained few cells other than polyploid cells, the highest level attained being 32c. During decidualization, there was a progressive increase in labelling with [³H]thymidine and in mitotic indices up to the 3rd day. It is suggested that endopolyploidy results in this case from by-passing mitosis by cells lacking some rate-limiting metabolic factor indispensable to the mitotic phase.

INTRODUCTION

In most mammals during the progestational phase, the endometrial stroma becomes endowed with remarkable morphogenetic potencies. After the onset of ovo-implantation or following non-specific uterine stimulation, the endometrium undergoes decidual differentiation. This process is essentially characterized by the epitheloid transformation of stromal fibroblasts associated with activation of cell proliferation rate (see De Feo, 1967; Finn, 1971).

It has been shown that after 48 hr of development, decidual growth produces various endopolyploid nuclear populations (Sachs & Shelesnyak, 1955; Dupont, Duluc & Mayer, 1971; Zybina & Grishenko, 1972). The presence of such cells prevents the detailed study of mitotic parameters by classical methods based on the use of labelled thymidine, such as the fraction of labelled mitoses curve or double labelling methods. It therefore seemed advisable to study cell kinetics during decidualization not only by a single [³H]thymidine pulse but also through cytophotometric evaluation of DNA, which in this case appears to be a more suitable method.

MATERIALS AND METHODS

Animals and hormonal treatment

Rats of the Wistar R strain (average weight 170 g) were ovariectomized

under tribromoethanol (Avertin, Bayer Products) anaesthesia. After 10 days, they were submitted to a schedule of hormonal treatment based on the work of Psychoyos (1961) and Finn (1966) which is known to reproduce the endocrine conditions of the progestational phase.

This schedule (Text-fig. 1) consists of a daily injection of $0.6 \mu\text{g}$ oestradiol- 17β for 3 days followed by 24 hr without treatment, after which the animals received daily injections of 4 mg progesterone for 4 days. On the 4th day of progesterone treatment, $0.05 \mu\text{g}$ oestradiol- 17β was administered and, at laparotomy 12 hr later, the uterine horns were longitudinally scratched.

Preliminary experiments confirmed that this procedure elicits a massive decidual reaction within 4 days, provided that adequate hormonal support is maintained during this period. The latter treatment consisted of 2 mg progesterone with $0.2 \mu\text{g}$ oestradiol- 17β per day (Yochim & De Feo, 1962). Hormones were injected subcutaneously in oil.

Groups of six animals were killed at 24, 48, 72 and 96 hr after uterine trauma

TEXT-FIG. 1. Hormonal treatment and experimental programme applied for the study of DNA replication in the decidual cells of rats.

and their uteri were removed. In the control group, uterine horns were not scratched, and the animals were killed 24 hr after the injection of $0.05 \mu\text{g}$ oestradiol- 17β given on Day 8 of hormonal treatment.

Isolation of stromal and decidual nuclei

The method employed for isolating endometrial stromal nuclei was adapted from that described by Smith, Martin, King & Vertes (1970). The uterine horns of individual experimental groups were placed in ice-cold sucrose solution (0.25 M) supplemented with CaCl_2 (3 mM). The epithelium was removed from horns split longitudinally by shearing them between pestle and mortar of a Potter-Elvehjem homogenizer and the stroma was then separated from the myometrium by brief slow-speed treatment (250 rev/min for 30 sec) in an Ultra-Turrax homogenizer. After filtration through 100-mesh wire gauze, the stromal fraction was treated in a Potter-Elvehjem homogenizer (15 rev) in order to release the nuclei. This fraction was then submitted to centrifugation at 2000 g for 5 to 10 min which allowed elimination of the super-

nant fat. After refiltration in the initial medium through 200-mesh wire gauze and low-speed centrifugation (300 *g* for 5 to 10 min), the nuclear pellet was further purified by ultracentrifugation (30,000 *g* for 1 hr) through 2.3 M-sucrose supplemented with 1 mM-MgCl₂ and 2 mM-CaCl₂. The nuclei were resuspended in a few drops of the initial medium, smeared on gelatin-coated slides and fixed with neutral formalin under standard conditions.

After the 2nd day following uterine trauma, the decidual tissue was simply scraped from the myometrium and treated as described.

Cytophotometric method

A series of nuclear smears corresponding to the different stages of decidual reaction were simultaneously stained by Feulgen's method in Schiff's reagent after hydrolysis in 1 M-HCl at room temperature for 12 hr. Cytophotometric estimation of the staining was effected on individual nuclei with the micro-fluorimeter devised by Ploem (1967) and manufactured by Leitz (Wetzlar).

Tritiated thymidine autoradiography

In a second experiment, performed as outlined in the scheme shown in Text-fig. 1, animals received an intraperitoneal injection of 300 μ Ci [³H]-thymidine 20 min before being killed. A segment of uterine horn was fixed in Bouin's fluid for the preparation of histological sections from which to evaluate mitotic indices. The remaining uterine tissue was submitted to the procedure for the isolation of endometrial nuclei. Standard autoradiography with Ilford K₅ emulsion was performed on the corresponding nuclear smears. Autoradiographs were developed in 50% Dektol (Kodak) after 12 days exposure and were counterstained with Harris haematoxylin. This material served to establish the evolution of the [³H]thymidine labelling index during decidualization.

RESULTS

Feulgen cytophotometry

The irregular distribution of DNA per nucleus in the various experimental groups precluded statistical analysis. The data are therefore best presented as frequency histograms (Text-fig. 2). It can be seen that while decidual development progressed, new populations of nuclei with increasing DNA content were being produced.

The diploid population was most numerous and best defined in the control histogram, thereby allowing its mean Feulgen-DNA amount to be determined more easily than in other groups. With this aim, cumulated frequencies of control data were plotted against logarithms of DNA values (Text-fig. 3). The ascending limb of this curve showed an inflexion point corresponding to the diploid DNA level from which the higher degrees of ploidy could easily be determined.

At 24 hr after uterine trauma, a shift of the histogram towards higher DNA amounts occurred, some of the values being close to the octaploid level. This situation remained unchanged at 48 hr.

TEXT-FIG. 2. Frequency histograms of amounts of Feulgen-stained DNA in decidualizing cells of rats at various moments after uterine trauma. The total numbers of measurements in each group are given in parentheses. A.U.=arbitrary photometric units. For c, see text, p. 445.

After the first 2 days of decidual reaction, the production of endopolyploid nuclei was accelerated. At 72 hr, the tetraploid population was relatively much more numerous as indicated by the shoulder of the histogram at this level. At the same time, octaploid and even 16c (c: haploid DNA amount) values also appeared.

After 4 days, the decidual reaction was fully developed. The numerically most important group of cells engaged in this process appeared to be the tetraploid cells. There were also a few remaining diploid nuclei. A large increase of the octaploid population was indicated by the shoulder of the histogram

TEXT-FIG. 3. Cumulated frequency curve of nuclear Feulgen-DNA amounts in control rats, allowing definition of the diploid value (2c).

at this value. It also appeared that decidual endopolyploid nuclei could attain the 32c level.

In the frequency histograms corresponding to 72 and 96 hr of decidual development, there was considerable overlapping of DNA amounts representing the lower ploidy levels. Numerous intermediate values were scattered between the 8c, 16c and 32c levels.

Autoradiography and mitotic index

The data given in Table 1 indicate that mitotic as well as [^3H]thymidine-labelling indices steadily increased until the 3rd day of decidualization. This increase was followed by a drop of both parameters between 72 and 96 hr.

Table 1. Evolution of mitotic and [^3H]thymidine labelling indices in decidualizing endometrial cells in rats

<i>Hours after decidualizing stimulus</i>	<i>Labelling index (%)</i>	<i>Mitotic index (%) ($\pm$ S.E.M.)</i>
0 (controls)	3	1.5 ± 0.3
24	9	6.0 ± 0.2
48	32	9.4 ± 0.3
72	59	12.0 ± 0.3
96	34	3.4 ± 0.3

No S.E.M. could be estimated for the labelling indices since the data were obtained from pooled nuclear populations coming from groups of six animals.

DISCUSSION

To interpret our results, it must be recalled that the amount of nuclear DNA reflects the stage of division cycle in which a cell is engaged and that any phase of this cycle is represented by a number of corresponding DNA values which is proportional to its duration, provided that the population is asynchronous (Vendrely, 1971). The histogram of controls may therefore be regarded as characteristic of a proliferating diploid population. The major

TEXT-FIG. 4. Block diagram of cell proliferation kinetics during decidualization in the rat. See text (page 447) for explanation.

peak is composed of diploid nuclei in G_1 because this phase is by far the longest of the cycle. Around the tetraploid value, a few cells in G_2 phase are found, whereas the intermediate values represent the cells engaged in DNA replication (S phase).

The overlapping of values corresponding to the lower ploidy levels, as well as DNA amounts between 8c, 16c and 32c levels, correspond to the presence of an important proportion of cells engaged in DNA replication and therefore containing intermediate DNA quantities. The existence of large numbers of such cells is confirmed by the high [3H]thymidine-labelling indices found by other authors (Zhinkin & Samoshkina, 1967; Das & Martin, 1972) as well as ourselves under the same conditions.

In order to discuss our results in terms of cell proliferation kinetics, a block diagram (Text-fig. 4) will be used with the aim of integrating personal data as well as information available from the literature on decidual growth and cell kinetics in general.

The initial situation observed in controls is represented by a classical division cycle of a diploid cell population. The decidualizing stimulus would induce stromal cells of the endometrium to shift to another similar cycle endowed with a much higher proliferation rate. It is reasonable to assume that this stimulation occurs in G_1 phase since in most systems which have been studied, it is only at this particular stage of the cycle that cells can be induced to change their proliferation rate (see Cooper, 1971; Mueller, 1971).

From this accelerated circuit, three new cycles will successively be derived as decidualization progresses. At each of these three levels, a number of G_2 cells will by-pass the mitotic phase (M) to undertake another round of DNA replication and become polyploid. Possible variants would be to have polyploid cells stemming from an endomitotic process or directly derived from the preceding S phase without going through a new G_1 phase. Another fraction of G_2 cells on the contrary divides and therefore acts as feedback to the corresponding G_1 compartment. Indeed, chromosome counts performed by Sachs & Shelesnyak (1955) on decidual tissue squashes show that some of the polyploid nuclei do divide. We have therefore also introduced into the diagram several sources (S_1 to S_4) which are meant to account for cell doubling at the different levels and which are modulated by the output of the corresponding M compartment.

One of the features of the diagram is that it accounts for the 24- to 48-hr lag period before the acceleration of endopolyploidy production. Cells which have progressed in the initial cycle beyond the stage of G_1 phase suitable for decidual induction will have to travel through the entire cycle before returning in G_1 and shifting towards the more rapid cycle. For these cells, therefore, the sequence of compartments required to reach a higher ploidy level will be much longer than for those which could immediately be induced. In the former case, there will be seven compartments to go through ($S \rightarrow G_2 \rightarrow M \rightarrow G_1 \rightarrow S' \rightarrow G'_2 \rightarrow G''_1$) whereas in the latter, there will only be three ($S' \rightarrow G'_2 \rightarrow G''_1$). Therefore the production of polyploid nuclei will be accelerated in proportion to the initial cycle's draining into the second one. As can be seen in Text-fig. 2, there is a strong reduction in the number of diploid cells between Days 3 and 4. According to the diagram, this event would correspond to a

rapid clearance of the first induced cycle from the moment it is no longer fed by cells finishing their travel through the initial circuit.

The synthesis of DNA in decidualization, as shown by biochemical assays (Shelesnyak & Tic, 1963), is in good agreement with our data. During deciduoma formation, there is an exponential increase of DNA content in the uterus. During the first 2 days, DNA synthesis is only slightly stimulated. Later, it becomes accelerated to reach a sixfold increase of DNA in the total uterus within 4 days. This rise is almost entirely accounted for by the endometrial stroma where the increase of DNA must be at least 10-fold.

Some authors (see Dupont *et al.*, 1971) have suggested that decidual nuclei would arise from nuclear fusion in binucleate cells which can be found in the decidual tissue. Since no cytological evidence supporting this hypothesis is available, it appears, in view of the tremendous increase of DNA occurring within a short period, that such an uneconomical and time-consuming mechanism is unlikely.

An experimental system similar to decidualization is provided by regenerating liver after partial hepatectomy (see Bucher, 1963). In this case also, an intense cell proliferation associated with increasing endopolyploidy has been observed.

It is tempting to assume that because of the high speed of proliferation an increasing number of cells are lacking some rate-limiting metabolic factor which is indispensable to mitosis. Therefore, being supplied with everything necessary to accomplish the rest of the cycle, those cells would by-pass mitosis and become polyploid after another round of DNA replication. At any rate, it is clear that, contrary to events achieved during the G₁ and S phases (Mueller, 1971), the metabolic steps associated with mitosis are by no means compulsory for the pursuit of the cell cycle.

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