Proliferation of germ cells and somatic cells in first trimester human embryonic gonads as indicated by S and S+G₂+M phase fractions

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Abstract

Objectives: The number of germ cells and somatic cells in human embryonic and foetal gonads has previously been estimated by stereological methods, which are time- and labour-consuming with little information concerning cell proliferation. Here, we studied whether flow cytometry could be applied as an easier method, also enabling estimation of the fraction of cells in S or S+G₂+M (SG₂M) cell-cycle phases as indicators of cell proliferation.

Methods: Cell suspensions from 35 human embryonic gonads at days 37 to 68 post-conception (pc) were immunomagnetically sorted into C-KIT positive (germ) cells and negative (somatic) cells. They were stained for DNA content and analysed by flow cytometry. S and SG₂M fractions could be measured for 13 of the female and 20 of the male gonads. The number of cells was estimated using fluorescent reference beads.

Results: During the period from 37 to 68 days pc, female germ and somatic cells had a stable S and SG₂M fractions indicating steady growth of both subpopulations, whereas they decreased in both male germ and somatic cells. The number of germ and somatic cells estimated by flow cytometry was significantly lower than in stereological estimates, suggesting loss of cells during preparation.

Conclusions: Cell proliferation as indicated by S and SG₂M fractions could be estimated specifically for primordial germ and somatic cells. Estimation of total number of germ and somatic cells was not feasible.

Introduction

The genital ridges are formed when primordial germ cells colonize the ventral cranial part of the mesonephros that provides somatic cells to the ovary and testis (1,2). In the human, gonadal sex differentiation starts around week 6 to week 7 post-conception (pc) (3–5), and primordial germ cells are now called oogonia and prespermatogonia in the ovary and testis respectively. The major structural difference between ovary and testis is that pre-spermatogonia become enclosed in testicular cords together with Sertoli cells, whereas ovarian tissue remains rather homogeneous with oogonia distributed uniformly throughout the ovary (6,7). In the human ovary, a few germ cells will have entered the leptotene stage of meiosis already at around week 9 pc (7,8) whereby they lose their mitotic activity and become oocytes. In the embryonic testis, pre-spermatogonia are mitotically active after they become enclosed in the testicular cords.

Very few reports exist on numbers of primordial germ cells and somatic cells in human first trimester gonads. Using stereological methods, numbers of germ cells and somatic cells of human embryonic gonads have been determined by Bendsen *et al.* (6,7) and more recently by Lutterodt *et al.* (9) and Mamsen *et al.* (10), in good agreement with the data of Bendsen *et al.* From the 6th to 9th week of embryonic life, numbers of germ cells in ovaries were found to increase from ~5000 to ~150 000 cells and in testes from ~2000 to ~100 000 cells, whereas numbers of somatic cells in ovaries increased from ~100 000 to ~1 000 000 cells and in testes from ~100 000 to ~2 000 000.

Lutterodt *et al.* found that pregnant mothers' smoking tobacco during the first trimester significantly reduced numbers of ovarian somatic cells of the embryo/foetus as compared to non-smokers, and numbers of oogonia showed a tendency to be lower. Mamsen *et al.* found that smoking also affected embryonic testes. Here, numbers of both germ cells and somatic cells were significantly lower.

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Stereological methods are laborious and time-consuming, and faster counting methods would be welcomed as would estimates of levels of cell proliferation, for example, staining for Ki-67. However, histological sections used for stereological counting must be between 30 and 50 µm thick and are thus impermeable to most histochemical markers, such as Ki-67. A previous study used flow cytometry on nuclear suspensions of foetal mouse gonads to quantify cells with 2c DNA (G₁ phase cells), 2-4c DNA (S phase cells) and 4c DNA (G₂ phase cells as well as mitotic and meiotic cells), but these results were not verified by counting the cells in histological sections in gonads of similar ages (11). Other investigations have aimed at simplifying the process of evaluating numbers of germ and somatic cells by combining immunomagnetic sorting with DNA flow cytometry of gonad cell suspensions (12,13).

The aim of this study was to evaluate size and expansion of subpopulations of germ cells and somatic cells in gonads of first trimester human embryos, using combined immunomagnetic and flow cytometric techniques, on single-cell suspensions from the gonads. With this approach, we wanted to establish an easier method for estimation of numbers of germ cells and somatic cells in comparison to previously determined stereological data. As kinetic analysis based on labelling DNA synthesizing cells *in vivo*, for example with bromodeoxyuridine, is not ethically feasible, we considered S fraction or SG₂M fraction of momentary DNA content distribution, as the best possible indicator of level of cell proliferation.

Materials and methods

Tissue preparation

Human embryos were obtained from 40 legal abortions during their first trimester (6–12th gestational week, that is, 4–10th week pc) from women referred to the Department of Gynaecology and Obstetrics, Frederiksberg Hospital, Denmark. All participating women (aged 19–39 years) received oral and written information according to and approved by The Scientific Ethical Committee for the Capital Region (KF (01)258206). Embryonic age was evaluated before abortion by ultrasound, measuring crown rump length and verified after by measuring foot length, if possible (14). Sex of embryos was unknown to investigators involved.

Embryos were transported in sterile cups at 36 ± 1 °C, in Dulbecco's modified Eagles medium (DMEM) (Gibco Invitrogen, Taastrup, Denmark) and were dissected using a stereomicroscope within 3 h after they had been removed from the uterus. Gonads were isolated by dissection in saline. One gonad of each foetus was used for stereological evaluations of numbers of germ cells and somatic cells [female gonads (9); male gonads

(10)]. The other gonad from each patient was prepared into a single-cell suspension for immunomagnetic sorting. Five gonads were used for optimization of tissue disaggregation (two gonads) and evaluation of immunomagnetic sorting (three gonads). Thirty-five gonads were disaggregated in trypsin/EDTA solution [1 mM EDTA, 0.25% trypsin in M2 medium without Ca²⁺, Mg²⁺ and BSA (Gibco, Invitrogen)] for 5–6 min, and in PBS/DNAse solution (20 µg/ml deoxyribonuclease I) for 4–8 min, depending on size of the gonads. Cells were then resuspended in DMEM supplemented with 2 mM glutamax, 50 mg/l penicillin, 50 mg/l streptomycin and 10% foetal bovine serum, and were left to recover for 2 h at 37 °C. Unless otherwise mentioned, products were purchased from Sigma (St. Louis, MO, USA). A piece of somatic tissue of each embryo was snap frozen in liquid nitrogen, for determination of the embryo's sex, by a PCR technique (15).

Immunomagnetic sorting

Single-cell suspensions were labelled directly with magnetic beads conjugated with antibody against C-KIT (CD117) – a surface marker of primordial germ cells (2,16). All products and sorting procedures applied were according to manufacturers' instruction protocols [CD117 Microbead Kit, using microbeads conjugated to monoclonal anti-human CD117 antibody (clone AC126); Miltenyi Biotec, Bergisch Gladbach, Germany]. Briefly, cells were incubated in FcR blocking reagent and with microbeads conjugated with monoclonal anti-human CD117 antibody. Magnetically labelled cell suspension was applied to a magnetized MS column and allowed to run passively through it. Non-retained cells represent the C-KIT negative fraction. To retrieve the C-KIT positive fraction, the column was removed from the magnetic field, and retained cells were flushed out with the aid of a plunger. Sorted fractions were centrifuged and resuspended in 200 µl of buffer (250 mM sucrose, 40 mM trisodium citrate 2 H₂O, 5% v/v DMSO, adjusted to pH 7.6) before being frozen at –80 °C.

Efficiency of the immunomagnetic sorting was evaluated on a set of mouse embryonic gonads by flow cytometric analysis of CD117 expression of sorted cells, and on a set of three human embryonic gonads, by haemocytometer counts of unsorted and sorted cells, and by alkaline phosphatase (AP) staining of unsorted and sorted cells, centrifuged on to polylysine coated glass slides.

DNA analysis by flow cytometry

Frozen samples were thawed, centrifuged and stained with propidium iodide for DNA content according to the method of Vindeløv *et al.* (17). Stained nuclear suspensions were analysed in a FACSort flow cytometer (BD,

Franklin Lakes, NJ, USA), using propidium iodide fluorescence (FL2-H, band pass 585/42 nm) as threshold trigger. Gates in forward versus side scatter (FSC/SSC) and fluorescence pulse area versus height (FL2-A/FL2-H) were set to ensure that non-lysed cells and doublets were excluded from DNA content histograms. As the flow cytometer was not equipped with volumetric acquisition, an aliquot of 9000 Nile red beads (BD) was added to each sample before flow cytometry to enable calculation of total number of nuclei in the sample. DNA histograms were deconvoluted into cell cycle phase fractions according to the method of Vindeløv *et al.* (18), using zero degree polynomial for fitting the S phase fraction. Two gonads were excluded from the dataset as they displayed <1500 nuclear events (one female and one male). For one of the remaining gonads, total number of nuclei could not be estimated due to technical error (female gonad). Preservation of mitotic nuclei after staining *ad modum* Vindeløv *et al.* was validated by flow cytometry of U937 cells exposed to colcemid.

Statistics

Data were evaluated by regression analysis and paired *t*-testing relative to embryonic age. Flow cytometric estimates of numbers of cells were compared with stereological results in linear regression analysis on log-trans-

formed numbers of cells, relative to embryonic age, assuming exponential growth of gonad cell populations.

Results

Cell sorting

The immunomagnetic sorting procedure was tested on a set of mouse embryonic gonads (Fig. 1). Flow cytometry of the sorted cells, after staining with CD117 antibody against a further epitope than that of the CD117 antibody, which was conjugated to the paramagnetic microbeads, showed that CD117 expressing cells were the majority in the positive (retained) fraction, but efficiently depleted from the negative (not retained) fraction.

Alkaline phosphatase assay was applied to sorted cells from each of three human embryonic gonads (Table 1, Fig. 2). Alkaline phosphatase a marker for primordial germ cells, oogonia and prespermatogonia, was expressed on 2.3–3.4% of cells of the unsorted suspension, 65–86% of cells of the positive fraction, and on <0.1% of cells in the negative fraction. Combining haemocytometer counts with AP counts, recovery of AP-expressing cells in the positive fraction, compared to numbers of AP-expressing cells in the unsorted suspension, was calculated to be 89–133%.

Figure 1. Flow cytometry evaluation of immunomagnetic sorting of germ cells versus somatic cells. Cell suspensions were prepared from a set of mouse embryonic gonads (at 12½ days pc) and immunomagnetically sorted according to CD117 expression (clone AC126; Miltenyi Biotec). Sorted suspensions (except controls) were stained with phycoerythrin-conjugated anti-CD117 antibody (CD117-PE; clone A3C6E2, Miltenyi Biotec). Analysis of CD117-PE expression (lower panel) was performed on a subpopulation defined by forward and side scatter (R₁, upper panel). Estimates of CD117-PE positive fraction are indicated (R₅, lower panel).

Table 1. Content of germ cells in cell suspensions of human embryonic gonads as evaluated by alkaline phosphatase (AP) assay, before immunomagnetic sorting according to CD117 expression and in immunomagnetically positive and negative fractions

Gonad	Sample	Counted cells		AP+ cells	Total cells	Recovery
		AP+	AP-	(%)	AP+	(%)
Female gonad, 42 days pc (Pt no. 61)	Unsorted	46	1317	3.4	5287	
	Positive fraction	235	39	85.8	4700	89
	Negative fraction	1	1137	<0.1		
Male gonad, 42 days pc (Pt no. 63)	Unsorted	36	1511	2.3	3103	
	Positive fraction	280	151	65.0	2800	90
	Negative fraction	1	1950	<0.1		
Male gonad, 53 days pc (Pt no. 66)	Unsorted	76	2272	3.2	6560	
	Positive fraction	435	92	82.5	8700	133
	Negative fraction	2	3485	<0.1

Figure 2. Alkaline phosphatase (AP) expression of human embryonic gonad cells in the CD117-positive fraction from immunomagnetic sorting. Insert shows an AP positive cell at higher magnification. Male gonad, 53 days pc, patient no. 66.

DNA distribution

Examples of DNA histograms of immunomagnetically sorted germ cells (C-KIT positive) in Fig. 3a and somatic cells (C-KIT negative) in Fig. 3b show flow cytometric gating procedures and DNA measurement precision with regard to analysis of cell-cycle distribution. Median G_1 peak CV was 2.6% (range: 1.9–4.0%) for germ cells and 1.6% (range: 1.3–2.2%) for somatic cells. It is assumed that mitotic cells were represented in the DNA fluorescence histogram. This assumption is based on staining experiments on U937 cells exposed to colcemid (Fig. 4). A similar subpopulation of mitotic nuclei occurs after

staining *ad modum* Vindeløv *et al.* (17) as after staining with a previously established method for flow cytometric discrimination of mitotic cells (19).

Estimates of G_1 , S and G_2 +M cell cycle phase fractions for germ cells and somatic cells in female and male gonads are shown in Table 2 as range values for each week of embryonic age. For female germ cells and somatic cells, S and G_2 +M fractions were relatively stable with age, whereas S and G_2 +M fractions both decreased in coeval male germ cells and somatic cells. Ratios between S and G_2 +M fractions did not indicate any perturbation of cell cycle with embryonic age. The same pattern as for S and G_2 +M fractions became more evident when the sum of S and G_2 +M fraction (SG_2M) was examined in the same age groups.

In both female and male gonads, S phase fraction was significantly higher in germ cells than in somatic cells, as demonstrated by regression analysis relative to embryonic age (Fig. 5 and Table 3). Both for germ and somatic cells of female gonads, there was no significant change in numbers of cells in the S fraction during the entire period ($P = 0.63$ and $P = 0.56$ respectively), whereas germ cells and somatic cells of male gonads declined in number in the S fraction during the same period ($P = 0.0007$ and $P = 0.0001$ respectively).

Differences by regression analysis were more significant for the SG_2M fraction than for the S fraction. In both female and male gonads, SG_2M fraction was significantly higher in germ cells than in somatic cells (Fig. 6 and Table 3). For both germ cells and somatic cells of female gonads, there was no significant change in number of cells in SG_2M during the entire period ($P = 0.85$ and $P = 0.23$, respectively), whereas germ cells and somatic cells of male gonads declined in number in SG_2M over the same period ($P < 0.0001$ and $P < 0.0001$ respectively).

Figure 3. Flow cytometry analysis of cell cycle distribution in a human embryonic gonad at 42 days pc. (a) Germ cells. (b) Somatic cells. Gonad cell suspensions were immunomagnetically sorted into CD117 positive (germ) cells and negative (somatic) cells, stained with propidium iodide, and analysed by flow cytometry. DNA fluorescence histograms, gated in forward versus side scatter (R_1) and fluorescence pulse area versus height (R_2), were deconvoluted into cell cycle fractions (G_1 , S, $G_2 + M$). Nile red beads were added as internal reference for estimating numbers of cells.

Numbers of cells

Total number of cells in each sample stained for flow cytometry was estimated according to amount of Nile red beads added. In both flow cytometry and stereology, estimated number of somatic cells was significantly higher than number of germ cells; however, flow cytometry estimates showed almost no increase in cell number with embryonic age (Figs 7 and 8). Flow cytometry estimates

were highly significantly different from corresponding estimates by stereology (Table 3).

Discussion

DNA distribution

To our knowledge, this is the first study to evaluate the proliferation of germ cells and somatic cells in human first

Figure 4. Flow cytometry estimation of mitotic index in U937 cells. Cells of the histiocytic lymphoma derived cell line U937 were incubated with colcemid at 20 ng/ml for 4 h. All analyses from the same batch of cells. (a) Staining with propidium iodide *ad modum* Vindeløv *et al.* (17); mitotic nuclei (16%) represented by region P₂ in side scatter versus propidium iodide fluorescence dot plot. (b) Propidium iodide fluorescence histogram of the same sample as in Chart A; blue curve corresponds to region P₂ in Chart A. (c) Staining with propidium iodide after lysis with detergent Triton X-100 and subsequent fixation in formaldehyde *ad modum* Larsen *et al.* (19); mitotic nuclei (23%) are represented by region P₂ in side scatter versus propidium iodide fluorescence dot plot. (d) After lysis with detergent Triton X-100 and subsequent fixation in formaldehyde *ad modum* Larsen *et al.* (19) nuclei were additionally fixed in ethanol and sequentially stained with anti-phospho-histone H3 (pSer²⁸) (Sigma H9908), Alexa 488 conjugated secondary antibody (Invitrogen A11006) and propidium iodide *ad modum* Juan *et al.* (20,21); mitotic nuclei represented by blue dots, corresponding to region P₂ in Chart C.

trimester embryonic gonads by quantifying the DNA content distribution and analysing the distribution of germ cells and somatic cells in the different phases of the mitotic cycle.

Due to ethical considerations and because the study material was scarce and precious, methods for dynamic analysis of cell proliferation, by means of labelling of DNA synthesizing cells with tritiated thymidine or bromodeoxyuridine, or by stathmokinesis using mitotic arrest (22), could not be applied in this study. For lack of any better alternative, we considered S or SG₂M fractions of momentary DNA content distribution, as the best possible indicator of cell proliferation level. S or SG₂M phase fraction has been used elsewhere as a surrogate parameter for cell proliferation (proliferative index) in studies of human

tissues, for example, S phase fraction in human ovarian follicles (23), and SG₂M fraction in human epidermis (24), in human haematopoietic stem and progenitor cells (25) and in human breast cancer (26).

General guidelines for DNA flow cytometry advise against use of the SG₂M fraction because of artifacts, due to aggregates of G₁ phase cells wrongly interpreted as G₂+M cells (27); however, these guidelines mainly are aimed at flow cytometry analysis of fixed-cell suspensions. In this study, flow cytometry determination of DNA content in germ cells and somatic cells was performed using Vindeløv *et al.*'s staining method for unfixed cell suspensions (17,18). Thus, we assumed that resulting data would be representative of cell suspensions submitted for staining and provide a rapid and reliable estimate of the

Table 2. Cell-cycle distribution of germs cells and somatic cells in female and male embryonic gonads, relative to embryonic age

Age (days pc)	No. of ovaries	Female germ cells				Female somatic cells			
		G ₁ (%)	S (%)	G ₂ +M (%)	SG ₂ M (%)	G ₁ (%)	S (%)	G ₂ +M (%)	SG ₂ M (%)
35–41	2	72–76	16–19	8–9	24–28	91–92	6	2–3	8–9
42–48	5	71–75	17–21	7–10	26–28	91–94	4–7	2	6–9
49–55	2	74–79	13–18	7–8	21–25	92–93	5–6	2	7–8
56–62	2	71–73	18–20	9	27–29	91–92	7	1–3	8–10
63–69	2	72–75	16–21	8	24–29	92–96	4–6	0–2	4–8

Age (days pc)	No. of testes	Male germ cells				Male somatic cells			
		G ₁ (%)	S (%)	G ₂ +M (%)	SG ₂ M (%)	G ₁ (%)	S (%)	G ₂ +M (%)	SG ₂ M (%)
35–41	5	66–75	18–24	7–10	25–34	78–88	9–16	3–5	13–21
42–48	7	63–79	16–31	5–8	21–37	82–90	7–13	3–4	10–17
49–55	4	79–81	14–15	5–7	19–21	88–90	7–9	2–3	9–12
56–62	3	81–84	11–14	4–5	16–19	92–93	6	2	8
63–69	1	83	12	5	17	92	5	2	7

Figure 5. S phase fraction in female and male embryonic gonads relative to age in days pc. Female (■) and male (□) germ cells. Female (▼) and male (▽) somatic cells. Linear regression, 95% confidence limits.

cells' DNA content and distribution in the different cell cycle phases, with minimal artifacts, from G₁ doublets. Experiments in our laboratory have demonstrated that Vindeløv *et al.*'s staining method preserves the majority of mitotic nuclei, which is not always the case after use of other staining methods (Fig. 3).

Statistically, we considered sum of the S and G₂+M phase fractions (SG₂M) as a more accurate indicator of changes in the proliferative fraction of cells as it diminishes complementary errors in estimation of S and G₂+M compartments. This interpretation was not biased by cell-cycle perturbation complication as ratios between S and

G₂+M phase fractions were relatively stable (Table 2), and accumulation of DNA tetraploid cells in meiotic progression occurs later in embryonic development than the period included in this study.

Validity of our results from flow cytometry measurement of DNA distribution and number of cells may be limited if samples submitted to flow cytometry do not correctly represent the original populations of germ cells and somatic cells, after being processed through the preparatory steps of organ dissection, tissue disaggregation and immunomagnetic sorting according to C-KIT expression (11,12).

Analysis of DNA distributions revealed sex-specific difference in SG₂M fractions during the period analysed, suggesting a difference in level of cell proliferation (Fig. 6). Thus, female germ and somatic cells had stable SG₂M fractions, whereas SG₂M fractions in male germ and somatic cells were lower at the end of the period studied. Under the assumptions of representative samples, constant growth fractions and constant cell cycle phase durations, consequences of these findings would be that in female gonads, germ cells proliferate rapidly and somatic cells slowly at constant rates, throughout the studied time period, whereas in male gonads, germ cells and somatic cells proliferate at a decreasing rate towards end of the study time period.

Number of cells

The ratio between germ cells and somatic cells in human gonads during first trimester differs according to sex of the embryo under observation. Bendsen *et al.* (6,7) found that ratios between pre-spermatogonia and somatic cells

Table 3. Statistical analysis of number of germs cells and somatic cells in female and male embryonic gonads relative to embryonic age

	Categories compared	Regression analysis		
		Slope (<i>P</i> -value)	Elevation (<i>P</i> -value)	<i>t</i> -test, paired (<i>P</i> -value)
Figure 2	S fraction of female germ versus somatic cells	0.86	<0.0001	<0.0001
	S fraction of male germ versus somatic cells	0.43	<0.0001	<0.0001
	S fraction of germ cells, female versus male	0.017	—	
	S fraction of somatic cells, female versus male	0.0028	—	
Figure 3	SG ₂ M fraction of female germ versus somatic cells	0.60	<0.0001	<0.0001
	SG ₂ M fraction of male germ versus somatic cells	0.32	<0.0001	<0.0001
	SG ₂ M fraction of germ cells, female versus male	0.0009	—	
	SG ₂ M fraction of somatic cells, female versus male	0.0032	—	
Figure 4	Number of female germ cells, FCM versus stereology	0.0001	—	0.15
	Number of female somatic cells, FCM versus stereology	0.0035	—	0.002
	Number of female germ versus somatic cells, FCM	0.33	<0.0001	
	Number of female germ versus somatic cells, stereology	0.0008	—	
Figure 5	Number of male germ cells, FCM versus stereology	0.0004	—	0.55
	Number of male somatic cells, FCM versus stereology	0.0016	—	0.22
	Number of male germ versus somatic cells, FCM	0.84	<0.0001	
	Number of male germ versus somatic cells, stereology	0.41	<0.0001	
Figures 4 and 5	Number of germ cells, female versus male, FCM	0.81	0.92	
	Number of germ cells, female versus male, stereology	0.88	0.0082	
	Number of somatic cells, female versus male, FCM	0.53	0.67	
	Number of somatic cells, female versus male, stereology	0.014	—

Figure 6. SG₂M fraction in female and male embryonic gonads relative to age in days pc. Female (■) and male (□) germ cells. Female (▼) and male (▽) somatic cells. Linear regression, 95% confidence limits.

in the testis remained constant at about 1:44 during weeks 7–9, whereas ratios between oogonia and somatic cells in the ovary have a tendency to increase during week 6–9 from 1:9 to 1:6, respectively. Bendsen *et al.* also found that numbers of somatic cells in human testes from week

Figure 7. Number of germ cells and somatic cells in female embryonic gonads relative to age in days pc. Flow cytometry estimates of germ cells (●) and somatic cells (▲). Stereological estimates of germ cells (○) and somatic cells (△), data from Lutterodt *et al.* (9). Linear regression of log transformed data, 95% confidence limits.

7 to week 9 pc was 2–3 times higher than number of somatic cells in coeval ovaries, whereas number of germ cells in the ovaries was 3–4 times higher than number of

Figure 8. Number of germ cells and somatic cells in male embryonic gonads relative to age in days pc. Flow cytometry estimates of germ cells (●) and somatic cells (▲). Stereological estimates of germ cells (○) and somatic cells (△), data from Mamsen *et al.* (10). Linear regression of log transformed data, 95% confidence limits.

germ cells in testes. Lutterodt *et al.* (9) found that ratios between germ cells and somatic cells in the ovaries increased from 1:45 to 1:8 from 38 to 64 days pc (Fig. 7). Mamsen *et al.* (10) found that ratios between germ and somatic cells in testes increased from 1:68 to 1:39 from days 37 to 68 pc (Fig. 8).

When flow cytometry estimates of numbers of cells, in the present study, are compared to corresponding stereological estimates by Lutterodt *et al.* (Fig. 7) and by Mamsen *et al.* (Fig. 8), it is evident that there are significant differences between the results of these two methods. Flat curves of flow cytometry data mean that flow cytometry in its present form is useless as an alternative method for description of expansion in number of germ cells and somatic cells in embryonic gonads. To strengthen statistics for comparison, data sets included gonads from smoking as well as non-smoking mothers. For flow cytometry data on SG₂M fractions as well as number of cells, we found no statistical differences between smoking exposed and non-exposed subsets (data not shown).

Discrepancies between flow cytometry and stereological estimates of numbers of cells question validity of flow cytometry SG₂M fraction estimates, in particular, for male germ cells and somatic cells, where decrease in SG₂M fraction does not fit the stereologically observed increase in number of cells during the time period studied.

In the present study, numbers of and ratios between germ cell nuclei and somatic cell nuclei in the DNA stained samples, were estimated by applying a known number of Nile red reference beads to the cell samples

before analysing them by flow cytometry. This procedure did not take into account possible loss of material in the previous preparatory steps, such as dissection of embryos, enzymatic preparation of cell suspension, and immunomagnetic sorting. The reason why our estimates of number of germ and somatic cells in female and male embryonic gonads are discordant with corresponding data obtained using stereological methods is probably related to shortcomings of the tissue disaggregation procedure. In comparison to stereological data, it is evident that a large fraction of cells from older embryonic gonads are missing in samples analysed by flow cytometry. It seems that only certain amounts of cells were recovered for the final preparations. This applies to both germ cells and somatic cells, and both female and male gonads.

Conclusion

DNA flow cytometry of germ cells (C-KIT positive) and somatic cells (C-KIT negative) from human embryonic gonads at days 37 to 68 pc showed that during this period, female germ cells and somatic cells had stable S and SG₂M fractions, indicating steady growth of both subpopulations, whereas S and SG₂M fractions were lower in both male germ cells and somatic cells. Numbers of germ cells and somatic cells in cell suspensions available for flow cytometry was, however, significantly lower than number of cells determined by stereology. This discordance suggests loss of cells during preparation prior to flow cytometry, which may confound representativeness of the flow cytometric results.

We recommend that any future flow cytometry investigation of embryonic gonads (supposing legal permission and special medical training) focuses on improving the cell-suspension procedure and simplifying the entire preparative procedure. One approach might be to expand flow cytometry DNA analysis of nuclear suspensions prepared from immunomagnetically sorted cells, with addition of an empirical nuclear proliferation marker such as Ki-67 (28) or PCNA (29). Probably, the best approach would be dual fluorescence flow cytometry analysis of C-KIT and DNA. However, such analysis must be performed on a suspension of whole cells, not nuclei, and may hence be hampered by artefacts from cell aggregates or debris of damaged cells. Non-specific staining of DNA with propidium iodide, due co-staining of residual double-stranded RNA, may be alleviated by replacing propidium iodide with DAPI or Hoechst 33342, analysis of which, however, requires a flow cytometer with ultraviolet laser excitation. The most ambitious setup may include subvital incubation with bromodeoxyuridine, where dissected gonads would be slit to facilitate access of bromodeoxyuridine to proliferating cells of the interior.

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