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Original article

Induced differentiation of ovine foetal gonocytes after grafting in the scrotum of Nude mice

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Summary — This work was designed to elucidate, in foetal ovine testis, whether the reason why gonocytes do not differentiate into spermatogonia and do not initiate spermatogenesis is due to inadequate foetal environment (temperature and/or hormonal balance) or if somatic testicular cell proliferation is a prerequisite for initiation of male meiosis. The development and differentiation of gonocytes were analysed by grafting 60-day-old foetal ovine testes into the scrotum of immunotolerant adult Nude mice. Forty days after grafting, nine of the ten grafted testes had survived but had not increased in weight as compared to 60-day-old testes. Moreover, only one third of the graft was occupied by testicular tissue, in which the relative proportions of intertubular tissue and sex cords were not altered when compared with those of normal foetal testes. The remainer of the graft was occupied by teratoma. The total number of Leydig (–80%) cells, Sertoli (–66%) cells, gonocytes (–90%) and the total length of sex cords (–63%) per grafted testis were always significantly reduced ($P < 0.02$), whereas the sex cords were significantly increased in diameter (+36%; $P = 0.02$) as compared to those of non-grafted 60-day-old foetuses. However, in seven out of the nine testes, type A spermatogonia were obtained and in two of the seven a few type B or leptotene primary spermatocytes could be observed. The grafting of foetal testis in an adult scrotum induces differentiation of gonocytes into spermatogonia, independently of proliferation of Sertoli and Leydig cells.

testicle / foetus / graft / germ cell / somatic cell

Résumé — Induction de la différenciation des gonocytes par la greffe de testicules ovins foetaux dans le scrotum de souris Nude. Ce travail avait pour but de vérifier si l'absence de démarrage de la spermatogenèse dans le testicule foetal était la conséquence d'un environnement testiculaire inadéquat (température et/ou balance hormonale) ou si le démarrage de la spermatogenèse n'avait lieu qu'après la prolifération des cellules somatiques testiculaires. L'évolution des gonocytes a été analysée après greffe de testicules ovins foetaux de 60 jours de gestation dans le scrotum de souris Nude adultes immunotolérantes ; le délai entre la greffe et le prélèvement a été de 40 jours. Neuf des dix testicules greffés ont survécu mais ont seulement maintenu leur poids initial observé dans des foetus

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de 60 jours. De plus, seulement un tiers de la greffe est occupé par du tissu testiculaire dans lequel les proportions relatives de tissu intertubulaire et de cordons sexuels ne sont pas modifiées par rapport à celles de foetus normaux ; les deux tiers restants sont occupés par du mésenchyme et/ou un tératome complexe. Le nombre total de cellules de Leydig (– 80 %) et de Sertoli (– 66 %) et de gonocytes (– 90 %) et la longueur des cordons sexuels (– 63 %) par testicule greffé sont significativement ($p < 0.02$) diminués tandis que le diamètre moyen des cordons sexuels est significativement ($p = 0.02$) augmenté de + 36 % par rapport à celui des testicules non greffés de foetus de 60 jours. Cependant parmi les neufs testicules, sept présentaient des spermatogonies de type A et parmi les sept, deux avaient quelques spermatogonies de type B et des spermatocytes leptotène en petit nombre. La greffe de testicule foetal dans un scrotum adulte induit la différenciation des gonocytes en spermatogonies indépendamment de la prolifération des cellules de Sertoli ou de Leydig.

testicule / foetus / greffe / cellule germinale / cellule somatique

INTRODUCTION

In most mammals, the germ cells present in the foetal testis are gonocytes. These are large cells characterised by a prominent spherical nucleus with small nucleoli and spots of heterochromatin (Gondos, 1977). In the ovine, gonocytes have large spherical nuclei with small nucleoli associated with heterochromatin (Courot, 1962), whereas stem spermatogonia (type A) have an ovoid nucleus with a central prominent nucleolus and few if any heterochromatin. Among stem spermatogonia, two populations are observed: the reserve stem spermatogonia, referred to as A_0 spermatogonia with round-shaped nuclei and two to three nucleoli, and cyclic spermatogonia, referred to as A_1 to A_3 spermatogonia characterised by an ovoid nucleus and a central prominent nucleolus (Hochereau-de Reviers et al, 1976). Along with the spermatogonial divisions and the onset of the differentiation towards spermatogenesis, the spermatogonia and their nuclei decrease in size and become spherical (Courot et al, 1970; Clermont 1972). In some mammals at the end of gestation (cat: Ohno et al, 1962) or at birth (Meishan piglet: Hochereau-de Reviers et al, 1997), a few primitive spermatogonia or primary spermatocytes have been observed.

However, the grafting of a prepubertal mice testis into an adult scrotum or into an adult testis resulted in the rapid development of this grafted testis (Chan et al, 1969). This method, therefore, offers a way to test why the evolution of germ cells is blocked until the onset of pubertal testicular growth in mammals. To avoid rejection of the grafted testis, we have used as a recipient host, immunotolerant Nude mice, depleted in T lymphocytes. Rat spermatogenesis has been achieved in Nude mouse testes by injection of rat spermatogonia (Clouthier et al, 1996) in mouse Sertoli cell environment (Russel and Brinster, 1996).

This work was designed to elucidate, in foetal ovine testes, whether the reason why gonocytes do not differentiate into spermatogonia and do not give rise to spermatogenesis is due to either a higher body temperature than a scrotal one and because of a hormonal imbalance or to a prerequisite for testicular somatic cell proliferation before differentiation of germ cells.

MATERIAL AND METHODS

Booroola or Mérinos d'Arles blastocysts were transferred into pseudo-pregnant Mérinos d'Arles ewes, one blastocyst per female, to avoid the side-effects of the number of foetuses and to ensure the same genetic environment during pregnancy. The foetuses were then separated into two age groups before killing.

One testis per ovine foetus lamb was fixed at either 60 ± 1 ($n = 14$) or 100 ± 1 ($n = 10$) days of age. This tissue was from singleton pregnancies

and was fixed in Bouin-Hollande solution and processed as described previously (Hochereau-de Reviers et al, 1995). Paraffin sections (10 µm thick) were stained by Feulgen reaction for the nuclei of the testis cells and counterstained with Alcian blue.

Relative proportions of sex cords and intertubular tissue were determined with a 25-point ocular integrator (magnification × 320) on 20 fields per testis. Further, mean sex cord diameter was determined from 20 sex cord cross-sections per testis with a camera lens attached to a microscope (magnification × 100) and using a planimeter program (Apple II). The mean number of Sertoli and germ cells per cross-section of sex cord was determined from ten cross-sections per testis (magnification × 800). The relative proportion of the Leydig cells in the intertubular tissue was determined similarly with the ocular integrator (magnification × 800). The mean individual cross-sectional areas of gonocytes, Sertoli cell nuclei and Leydig cells were determined using the same planimeter program (magnification × 800).

Ten ovine foetal testes, dissected from the 60-day-old foetal lambs, were grafted into the scrota of intact adult Nude males, closed to the host's left testis, inside the tunica vaginalis. Nude mice were used as the host of these grafts since xenografting does not result in the rejection because of the immune incompetance due to absence of T Lymphocytes. Nine grafted testes survived and were adequately vascularised. They were killed 40 days after grafting. Grafted testes and testes of the host mice were dissected and weighed. Grafted testes were fixed and processed for histological analysis as decribed previously. The results were compared using the Student's *t*-test or by a one-way variance analysis.

RESULTS

Evolution of normal testis growth in the foetus (table I).

The testes increased in weight eight-fold between 60 (14.8 ± 2.7 mg) and 100 days (123.7 ± 22.3 mg) of gestation. The relative proportion of the sex cords in the testicular parenchyma did not vary between 60 and 100 days of age and the intertubular tissue represented more than half of the testis parenchyma (sex cords = 46%; intertubular tissue = 54%). The sex cords grew in length (× 9) between 60 and 100 days of age (60 days = 3.3 m; 100 days = 30 m), but their mean diameter (40 µm) did not vary during this period. The total number of Sertoli cells per testis increased, by a factor of 10 between 60 and 100 days of gestation (60 days = 3.5×10^6; 100 days = 35.6×10^6) similar to the total length of sex cords. Concommitantly, the germ cells, ie the gonocytes, increased by only a factor of 3.2 during the same period (60 days = 0.7×10^6; 100 days = 2.4×10^6). In the intertubular

Table I. Composition of normal or grafted foetal ovine testis (m ± sd).

Age (days)	60	60 + 40	100
Testis weight (mg)	14.8 ± 2.7 a	10.4 ± 4.5a	123.7 ± 22.3b
Sex cord relative proportion (%)	45.8 ± 4.2a	17.7 ± 14.7*	47 ± 4.1a
		39.9 ± 19.2a**	
Sex cord mean diameter (µm)	40.7 ± 3.5	54.1 ± 12.2	39.9 ± 3.1
Sex cord total length per testis (m)	5.2 ± 1.1a	1.4 ± 1.6b	46.7 ± 8.5c
Total number of Sertoli cells per testis (10^6)	5.6 ± 0.9a	1.9 ± 2.2b	556 ± 145c
Total number of germ cells per testis (10^6)	1.1 ± 0.3a	0.150 ± 0.119b	3.8 ± 0.6c
Total number of Leydig cells per testis (10^6)	2.3 ± 0.5a	0.8 ± 1.1b.	35.1 ± 11.0c
ASpgonia/germ cells (%)	0	31 ± 36	0

a, b and c superscripts indicate significantly different values. * Proportion calculated in the total grafted testis. ** Proportion calculated in the testicular part of the grafted testis.

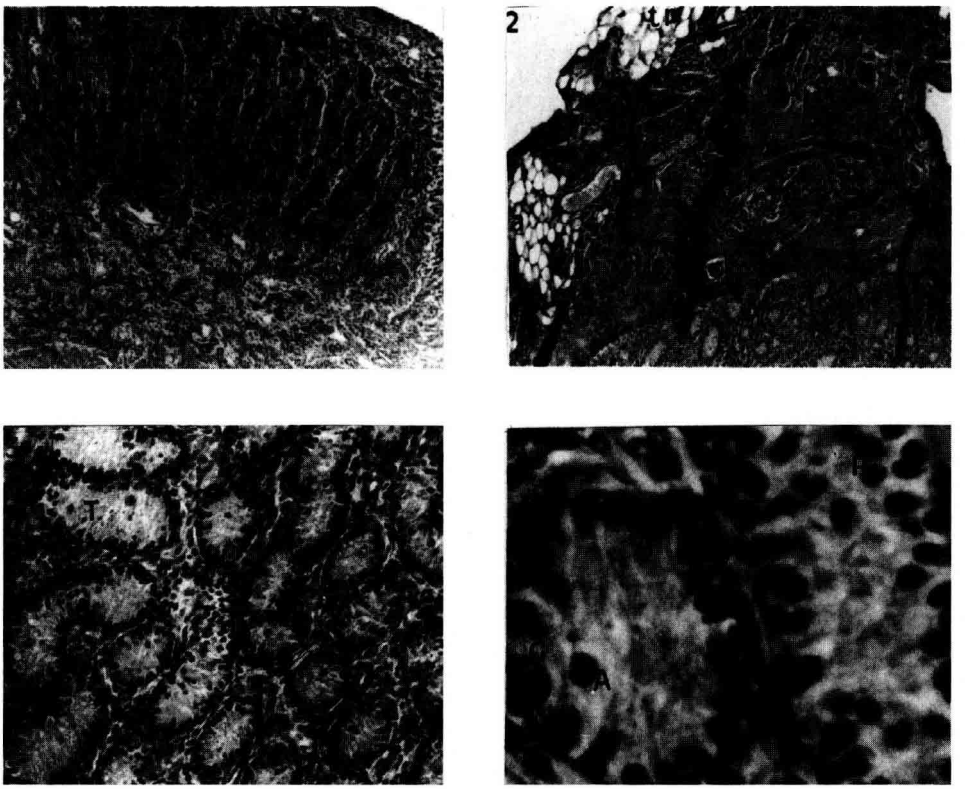

Fig 1. Cross-section of a normal 100-day-old ovine foetal testis. Note the long sex cords (T) joining the rete testis (R). Future Sertoli cells are present at the periphery of the sex cords and 30% of the sex cord volume is occupied by the gonocytes. Leydig cell islets are present between the cords. **2.** Section of a 60-day-old ovine foetal testis 40 days after grafting into the scrotum of a Nude mouse. Note that the upper part of the section is occupied by teratogenic (tr) tissue containing adipose tissue (a), muscle fibres (m), cartilage (C), blood (o) and epithelial tissue (e). **3.** Section of a 60-day-old ovine foetal testis 40 days after grafting into the scrotum of a Nude mouse. Note some of the sex cords appear enlarged (T) as compared with normal sex cords (t) and contain few spermatogonial cells. **4.** Section of a 60-day-old ovine foetal testis 40 days after grafting into the scrotum of a Nude mouse. Note the large gonocytes (G) with their prominent heterochromatic nuclei, the Sertoli cells (S) with their elongated nuclei, two type A spermatogonia (A) with their ovoid nuclei smaller than gonocyte nuclei and their central nucleolus and a few type B and preleptotene primary spermatocytes (P).

sented a higher production of primary spermatocytes than the littermate controls (Boujrad et al, 1995). The effect of a high internal temperature alone can not be considered as the reason for the blocking of meiosis appearance.

The grafted ovine foetal testes appeared to be well vascularised by 40 days after surgical grafting, but they did not grow in size. This could be interpreted as a survival process rather than a developmental one. Moreover, the composition of the grafted testis

was relatively different from that of the 100-day-old foetal testis. The fact that the grafts were placed so as to be closed to the testis and not inside the tunica albuginea of the host testis probably limited the vascularisation and the development of the graft. More than half of the graft was occupied by non-testicular tissue, containing mainly fibroblasts, but also bones, muscles, adipose tissue, skin and epithelia. Such cells and tissues were not found in 60-day-old foetal testes. It could be hypothesised that some pluripotent cells remain in the foetal testis, and are able to differentiate further. When 30-day-old foetal piglet gonocytes were injected into the Nude mouse testis, we obtained very little colonisation of seminiferous tubules of mice, although a large teratoma did develop (Hochereau-de Reviers and Perreau, pers comm). However, even though this occurred at a low efficiency, the onset of spermatogenesis was observed in few sex cords of the grafted testis. The grafting of foetal ovine testes or the injection of foetal piglet gonocytes in adult mice scrotum reveals two major facts: some pluripotent cells are still present in the ovine testis and differentiation of gonocytes toward spermatogonia do not require prerequisite proliferation of neighbouring somatic cells.

However, the efficiency of the differentiation processes are poor and few spermatogonia and primary spermatocytes are observed in grafted foetal ovine testes in the current experiment. This could originate from species interactions. In most cases, the transplanted rodent testis has been grafted into a rodent host: either in isogenic rats or between different species of rodents. The transplantation of new born rat testis under the kidney capsule of adult host rat of the same strain was developed as a model of Leydig cell development. In these models, the grafted testis proved to be steroidogenically active and responsive to gonadotrophin stimulation (Kuopio et al, 1989). Reciprocal translocation of testes from the scrotum of normal or pseudohermaphroditic male rats into the ear has also been successfull (Chan et al, 1969). In these cases, all the testes survived and gave rise to all stages of spermatogenesis normally seen in the different genetic strains, with the pseudohermaphroditic rats being blocked at the same stage as in the homologous non-grafted males (Chan et al, 1969). Goldstein et al (1983) also performed complex testis transplants, which survived for 12 weeks or more under the kidney capsule. The functional capacity of these transplants was judged to be normal as assessed by their production of androgen and androgen binding protein. Also the intertubular tissue and Leydig cell functions appeared to be maintained as normal. To assess the quality of grafts into male castrated recipient hosts, a long-term survey of gonadotrophin and testosterone plasma levels was undertaken (Gittes et al, 1972). The increase in FSH and the decrease in testosterone peripheral plasma levels suggested that the transplanted tissue was in fact partly regressed or dysfunctional. The hormonal peptide secretions of the host testis could influence the grafted gonad development. Grafting of mice foetal ovaries at day 12 of gestation under the kidney capsule of adult males of the same strain was performed and analysed 4 and 14 days later (Taketo et al, 1984). In grafted gonads 14 days post surgery, some sex cords could be observed and they were MIS (Müllerian inhibiting substance) and SGP-2 (clusterin) positive (Taketo, 1991), these secretions being associated with maleness and probably sex-reversal of the graft by the hormonal status of the host. Johnson et al (1996) transplanted testes from various donors at different ages (pre/postnatally) and compared the number of Sertoli cells between normal and transplanted testes. Despite an initial delay to reach the adult numbers of Sertoli cells, the developmental pattern of Sertoli cell proliferation was similar in the transplanted testis to a normal testis (Johnson et al, 1996). In grafted rodent testes, Sertoli and germ cells proliferate in the sex cords.

When neonate rat testes were implanted into adult testes, their development was delayed as compared with non-transplanted testis. However, in a large part of the transplant, spermatogonial divisions, meiotic prophase and spermiogenesis were observed (Luxembourger and Aron, 1966). Murine male germ cell transplantation has been successfully performed either by direct injection into the seminiferous tubules (Brinster and Zimmerman, 1994; Russell and Brinster, 1996) or by injection into the rete testis (Jiang and Short, 1995). This resulted in the colonisation and synchronisation of both host and transplanted cells (Jiang and Short, 1995), even when interspecies transplantation was performed (Clouthier et al, 1996; Russel and Brinster, 1996).

A species-dependent effect of grafting could be inferred. Human foetal gonads were grafted into the abdominal wall of adult Nude mice. After 40 days, gonocytes were still present, whereas the same approach performed with 3-day-old rat testis gave rise to differentiated germ cells (Skakkebaek et al 1974). This could be interpreted as a species-dependent difference.

In the current experiment, the number of Leydig cells decreased, suggesting that there could be a low intratesticular testosterone concentration. The decrease in Leydig cells could participate in the occurrence of a low yield of spermatogonial differentiation. However, a decline in Sertoli cell populations was also observed after grafting in the current experiment. In terms of sex cord development and Sertoli cell proliferation, the total initial population of Sertoli cells, per grafted testis, was not maintained, indicating that either Sertoli cells initially multiply and then degenerate or stop dividing. The pattern of differentiation from gonocytes to spermatogonia and meiotic prophase was comparable to that observed in normal lambs (Monet-Kuntz et al, 1984) with, first, the appearance of A_0 spermatogonia, followed by A_{1-3} spermatogonia, intermediate and type B spermatogonia and leptotene primary spermatocytes. The 40-day period was probably too short to ensure more progression in all of the grafted testes.

In conclusion, the surgical grafting of an ovine foetal testis into an immunosupressed mouse scrotum did not maintain normal testicular growth with increases in Sertoli, Leydig and germ cell numbers. However, some of the differentiation processes of gonocytes toward type A spermatogonia and spermatogenesis were initiated. This indicates that somatic cell proliferation is not a prerequisite for initiation of male meiosis eventhough the total number of Sertoli cells and the daily production of primary spermatocytes per testis were highly correlated (Hochereau-de Reviers et al, 1987).

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