Feminizing Effect of Mesonephros on Cultured Differentiating Mouse Gonads and Ducts

Abstract. *Gonads were removed from fetal mice at about the time that gonadal sex differentiation occurs. The gonads were cultured in vitro with or without their mesonephric tissue. When gonads and ducts removed from sexually undifferentiated fetuses were cultured together, the gonads of both sexes developed female characteristics, whereas gonads cultured without mesonephros developed according to the sex of the fetus from which they were removed. Gonads of sexually differentiated fetuses developed normally whether they were cultured with or without the mesonephros.*

In mammals, gonadal sex differentiation is generally believed to be influenced primarily by the chromosomal endowment. The undifferentiated gonad resembles morphologically a young ovary, and masculinization must be imposed on it if it is to escape feminine development (*1*). It is not known how maleness is imposed on the undifferentiated gonad. Neither sex steroids (*2*) nor gonadotropins (*3*) seem to act in mammalian gonadal sex differentiation. However, if undifferentiated mouse testes are cultured in media in which epididymis of fetal mouse (*4*), pubertal bull (*5*), or fetal and adult human epididymis (*6*) have been grown previously, the morphology of these testes is partly or completely feminized after 5 to 7 days. The testicular cords are absent and the male germ cells are found to have entered meiosis, suggesting that the mesonephric-derived tissues secrete a meiosis-inducing substance (*4–6*). We have studied in vitro the interaction of the mesonephros with the gonad of the fetal mouse, and have found that the fetal undifferentiated mouse testis as well as the reproductive ducts (Wolffian and Müllerian) develop feminine characteristics when the testis is cultured together with its mesonephric tissues.

To determine the stage at which the feminizing influence of the mesonephric tissues develops, gonads with the attached mesonephric tissues were removed from fetal mice (Bagg Albino) on days 10, 11, 12, 12½, 13, and 14 after the copulatory plug was found (day 1 is the day of plug). A more precise dating of the fetal age was obtained by photographing the hind leg of the fetuses and comparing the size of the leg with a dating scheme (*7*). From each fetus one gonad was cultured together with its attached mesonephric tissues (g+m); the other gonad was separated by dissection from the mesonephric tissue and cultured alone (g). A complete separation of gonad and mesonephric tissues was not possible in any of the day 10 fetuses, nor in some of the fetuses at days 11, 12, and 12½. These incompletely separated gonads were termed g+(m) (see Table 1). The gonads were cultured for a week and processed for histology as described elsewhere (*5*).

In mice, gonadal sex cannot be determined morphologically under the dissecting microscope before day 12½. We therefore examined histological sections of all the cultured gonads for sex-determination. If testicular cords with prespermatogonia were present in one of the cultured gonads, this was judged to be the gonad of a male fetus. If no such signs of testicular differentiation could be traced, the gonad was considered to be that of a female fetus (except gonads from day 10 fetuses, see below). The sex of gonads from fetuses removed on day 12½ or later can be determined under the dissecting microscope (*8*).

The number, sex, and degree of separation from mesonephric tissues of the cultured gonads are given in Table 1. All the gonads removed from day 10 fetuses lacked signs of testicular cords and contained germ cells in meiosis [leptotene (L), zygotene (Z), and pachytene (P)]. Some of these gonads are probably feminized testes because it seems unlikely that all the 20 fetuses (40 gonads) were females. The number of germ cells was much lower than in cultures of older gonads. Nonmeiotic germ cells could not be recognized with certainty. As one would expect for a female pattern of differentiation of the reproductive ducts, mitotic cells were present in the Müllerian duct. The Wolffian duct could not be recognized, whereas mesonephric tubules with mitotic cells were seen.

All of the ovaries removed from fetuses on day 11 or later, and cultured with or without the mesonephros, contained germ cells in meiosis. The number of germ cells was highest in the older fetuses and higher in g+m ovaries than in g ovaries of the same age. The differentiation of the ducts followed the female pattern, with a growing Müllerian duct and a degenerating Wolffian duct. The development of mesonephric tubules was also evident.

All of the g+m testes removed from fetuses on day 11, but only eight of the 11 g+m testes removed on day 12 became completely feminized, that is, morphologically indistinguishable from cultured female gonads. None of the older g+m

Table 1. Feminization of fetal mouse gonads in culture. Partial or incomplete separation of gonad (g) from mesonephros (m) is indicated by g+(m). All female gonads developed in a feminine way.

Fetal age at start of culture (days after copulatory plug)	Separation of gonads from mesonephros	Morphological sex at start of culture: Male — Morphological sex after culture: ♂	♂♀	♀	Male — Total number of gonads (and fetuses)	Female — Total number of gonads (and fetuses)
10	g					
10	g+(m)			*	*	40*
10	g+m					(20)
11	g	4				
11	g+(m)		10		28	32
11	g+m			14	(14)	(16)
12	g	6				
12	g+(m)		5		22	26
12	g+m		3	8	(11)	(13)
12½	g	8				
12½	g+(m)	1	1		20	18
12½	g+m	1	9		(10)	(9)
13	g	10				
13	g+(m)		0		20	20
13	g+m	10†			(10)	(10)
14	g	10			20	20
14	g+(m)		0		(10)	(10)
14	g+m	10				

*All gonads from day 10 fetuses contained various amounts of mesonephric tissue. They showed germ cells in meiosis, and no testicular cord formation. †Parts of (g+m) testes from day 13 fetuses showed impaired formation of testicular cords and prespermatogonia lying freely within the gonadal tissue. However, no germ cells were in meiosis.

0036-8075/81/0515-0817$00.50/0

testes became so feminized. Three g+m testes removed from fetuses on day 12 and nine g+m testes removed on day 12½ became partly feminized, having germ cells in meiosis (L, Z, and P) as well as prespermatogonia in testicular cords. One g+m testis from a day 12½ fetus differentiated as a normal male. None of the g+m testes from day 13 fetuses contained germ cells in meiosis, but in some cultures the testicular formation was impaired and the prespermatogonia were lying freely within the gonadal tissue. All g+m testes of day 14 differentiated normally with prespermatogonia in well-defined testicular cords.

All g+(m) testes from day 11 and day 12 fetuses were partly feminized. One g+(m) testis from a day 12½ fetus was also partly feminine, whereas the other differentiated into a testis. All g testes of day 11 and of older fetuses developed without feminization.

In cultures of g+m obtained from male fetuses on days 11, 12, and 12½, almost all the reproductive ducts became feminized to varying degrees. All those from day 11 and most of those from day 12 fetuses showed developing Müllerian ducts with evidence of mitosis and degenerating Wolffian ducts. Those that were less feminized (from day 12 and 12½ fetuses) showed Müllerian and Wolffian ducts that often included both degenerating areas and mitotic cells. In g+m testes showing no feminization the Müllerian duct was degenerating and the Wolffian duct growing. The mesonephric tubules grew in all cultures regardless of the degree or absence of feminization.

The feminizing effect of the mesonephric system on testicular differentiation in cultured gonads of fetal male mice was described previously (*4–6*). The feminizing influence was also observed in fetal mouse hermaphrodites (*9*) and in fetal sex-reversed XY mouse gonads (*10*), in which the ovarian parts of the gonads almost always were found close to the mesonephric system.

An H-Y antigen has been described (*11, 12*) as the primary testicular organizer in vivo. If the H-Y antigen also affects testicular differentiation in vitro, the present results imply that mesonephros antagonizes this substance, because male gonads cultured together with the mesonephros become feminized.

Our results indicate that anti-Müllerian hormone (*13*) does not function or is not secreted in the feminized testis in vitro. This seems reasonable since the anti-Müllerian hormone probably arises from the Sertoli cells, which do not differentiate when testicular cords are absent.

Radioimmunological studies indicate that less testosterone is present in culture media from feminized testes (g+m) than in media from testes separated from the mesonephros (g). This is in agreement with data for cultured fetal rabbit gonads that show that media from testes cultured with mesonephros contain less testosterone than media from testes cultured alone (*14*). However, studies of fetal mouse gonads in vitro show that the addition of testosterone does not overcome the feminizing influence of mesonephros (*15*).

This study demonstrates that the Wolffian and Müllerian ducts interact with the developing gonad and that sex differentiation of gonads and ducts depends on this interaction rather than on the chromosomal endowment alone.

It also shows that the degree of testicular feminization is inversely correlated to masculinization of the reproductive ducts.

ANNE GRETE BYSKOV
JØRGEN GRINSTED
Finsen Laboratory, Finsen Institute, Strandboulevarden 49, 2100 Copenhagen Ø., DK-Denmark

References and Notes

1. A. Jost, *Adv. Biosci.* **10**, 3 (1972).
2. K. Haffen and E. Wolff, in *The Ovary*, L. Zuckerman and B. J. Weir, Eds. (Academic Press, New York, 1977), pp. 393–446.
3. P. Mauleon, in *Reproduction in Domestic Animals*, H. H Cole and P. T. Cupps, Eds. (Academic Press, New York, 1969), pp. 187–215.
4. A. G. Byskov, *Int. J. Androl. Suppl.* **2**, 29 (1978).
5. J. Grinsted, A. G. Byskov, M. P. Andreasen, *J. Reprod. Fertil.* **56**, 653 (1979).
6. J. Grinsted and A. G. Byskov, *Fertil. Steril.*, in press.
7. R. Rugh, *The Mouse* (Burgess, Minneapolis, 1968).
8 A. G. Byskov, in *Mechanisms of Sex Differentiation in Animals and Man*, C. R. Austin and R. E. Edwards, Eds. (Academic Press, New York, in press).
9. W. K.Whitten, W. G. Beamer, A. G. Byskov, *J. Embryol. Exp. Morphol.* **52**, 63 (1979).
10. A. McLaren, *J. Reprod. Fertil.* **61**, 461 (1981).
11. S. Ohno, *Major Sex Determining Genes* (Springer-Verlag, New York, 1979).
12. S. S. Wachtel and G. C. Koo, in *Mechanisms of Sex Differentiation in Animals and Man*, C. R. Austin and R. E. Edwards, Eds. (Academic Press, New York, in press).
13. N. Josso, J. Y. Picard, D. Tran, *Recent Prog. Horm. Res.* **33**, 117 (1977).
14. J. Grinsted, A. G. Byskov, I. J. Christensen, J. C. Jensenius, in preparation.
15. C. Y. Andersen, A. G. Byskov, J. Grinsted, in preparation.
16. We thank Jette Lise Hansen, Inge Husum, Anne Lise Mohr, and Lene Ahrenst for technical assistance. This investigation was supported by Danish Medical Research Council grants 512-10639 and 512-20059, the Nordic Insulin Foundation, and EURATOM contract 120-73-1 BIO-DK.

17 September 1980; revised 23 February 1981

Hormonal Requirements for Growth of Arterial Smooth Muscle Cells in vitro: An Endocrine Approach to Atherosclerosis

Abstract. *In this study the hormonal requirements for the growth of arterial smooth muscle cells in vitro were determined. A serum-free, biochemically defined medium, supplemented with the relevant hormones, permitted proliferation and propagation of normal diploid mammalian arterial smooth muscle cells. Serum-free, hormone-supplemented cultures spontaneously formed atherosclerotic plaque-like nodules. Thus atherosclerosis may be mediated by a complex endocrine system.*

In rats, hypophysectomy can inhibit the proliferation of arterial smooth muscle cells that occurs in response to aortic de-endothelialization (*2*). This indicates that pituitary-derived as well as platelet-derived (*1*) hormones regulate arterial smooth muscle cell migration and proliferation in vivo. To elucidate these regulatory influences on the vascular smooth muscle cell would permit a better understanding of atherosclerosis as a failure to control smooth muscle cell proliferation (*3–6*). The importance of hormones in cell proliferation has been established by the demonstration that serum-free media, supplemented with specified hormones, permit the proliferation and propagation of several established cell

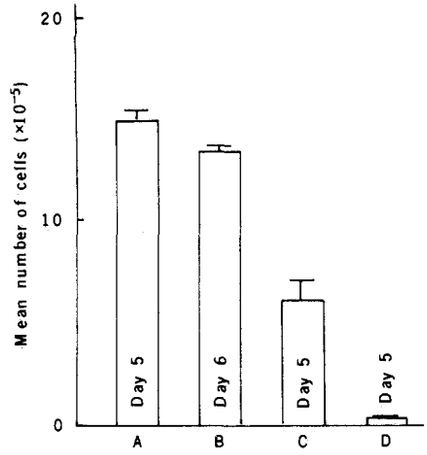

Fig. 1. Growth of rat abdominal aortic smooth muscle cells cultured in MEM or DMB. The cells were harvested from a serum-fed stock plate as described in the legend to Table 1. Duplicate 35-mm culture wells were coated with fibronectin (10 μg/cm²) and the cells were inoculated (10^4 per well) and fed MEM plus 5 percent FBS (A), DMB (B and C), or 1 percent FBS (D). Serum-free and hormone-free cultures were dead by day 4.

0036-8075/81/0515-0818$00.50/0