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.

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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

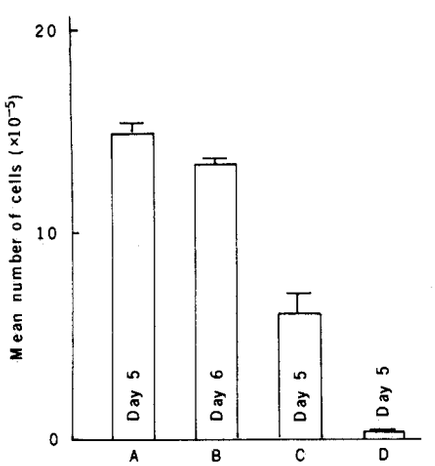

Fig. 1. Growth of rat abdominal aortic smooth muscle cells cultured in MEM or DMB. The cells were harvested from a serum-fed stock plate as described in the legend to Table 1. Duplicate 35-mm culture wells were coated with fibronectin (10 μg/cm²) and the cells were inoculated (10^4 per well) and fed 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.

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lines in culture (*7–13*). This dependence on hormones is shared by normal, diploid mammalian cells (*13–15*), whose growth is strongly promoted by such hormone-like substances as nerve growth factor (NGF) (*16*), epidermal growth factor (EGF) (*17*), platelet-derived growth factor (PDGF) (*2, 18*), and endothelial cell growth factor (ECGF) (*19*). We have now determined the hormonal requirements of rat abdominal aortic smooth muscle cells for growth and propagation in vitro. The identified hormones can be substituted for serum in cell culture and permit the proliferation and subculture of the cells.

Abdominal aortic smooth muscle cells were removed from Sprague-Dawley (Zivic-Miller) rats (*20*). Stock cultures were grown in Eagle's minimum essential medium (MEM) (Dulbecco's modification) and 5 percent fetal bovine serum (FBS), and the medium was changed every 2 to 3 days. Experimental culture plates were first coated with human fibronectin (10 $\mu g/cm^2$) made from human Cohn fraction I (*21*) to minimize plating efficiency as a variable contributing to increase in cell number. Cells were plated at a density of 1.25×10^3 per square centimeter per dish in MEM and the minimal amount of FBS (0.5 to 1 percent) that would permit survival without proliferation. The cultures were then supplemented with a given hormone and monitored for changes in cell number.

As indicated in Table 1, PDGF, EGF, insulin, transferrin, insulin-like growth factor (IGF-I), multiplication stimulating activity (MSA), hydrocortisone, and human α-thrombin were growth-promoting. A serum-free medium, designated DMB, was then formulated consisting of these hormones in a 1:1 (by volume) mixture of MEM and Ham's F12 supplemented with ovalbumin (1 mg/ml), ascorbic acid (25 μg/ml) (*22*) and trace elements (*8, 23*). [Partially purified fractions from human Cohn fraction IV (*24*), which is inexpensive and readily available, can be substituted for the more expensive somatomedins MSA (*25*) and IGF-I (*26*).] The ability of this serum-free medium to support smooth muscle proliferation is shown in Figs. 1 and 2. Ham's F12 was used to provide a more nutritionally complete medium than could be provided with a basal medium such as MEM alone. Although serum-fed cells plated at near-clonal density reached confluence faster than cells grown in DMB (Fig. 1), stock cultures maintained in DMB could be passaged at nearly the same frequency as serum-fed stocks. Our serum-free stocks were in their seventh passage at the time this report was written.

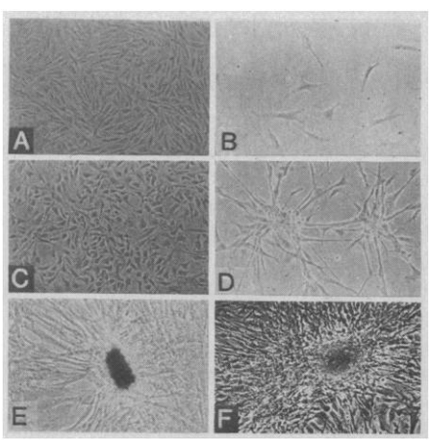

Fig. 2. Phase-contrast photomicrographs (×100) of subcultured smooth muscle cells from rat abdominal aorta and from human umbilical artery. (A) Rat cells cultured in MEM plus 5 percent FBS (day 5); (B) rat cells cultured in MEM plus 1 percent FBS (day 5); (C) rat cells cultured in DMB (day 5); (D) polypoid nodules in rat cells cultured in DMB, confluent density; (E) polypoid nodule in human cells cultured in MEM plus 5 percent FBS; and (F) polypoid nodules in rat cells explanted into MEM plus 5 percent FBS.

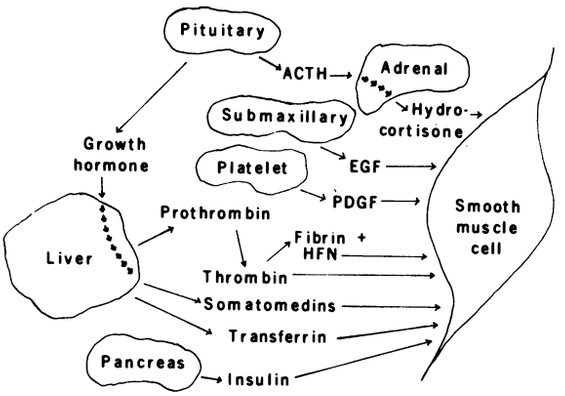

Fig. 3. Influence of hormones on arterial smooth muscle cell growth: an endocrine approach to atherosclerosis. Depicted are the hormones required for smooth muscle cell growth and their parent organs, *ACTH*, adrenocorticotropic hormone.

On reaching confluence, cultures grown in FBS exhibited the characteristic overlapping pattern of medial smooth muscle while cultures grown in DMB exhibited an exaggeration of the "hill and valley" pattern usually seen in medial smooth muscle cells grown in homologous serum (*27, 28*) (Fig. 2). Less than 24 hours after reaching confluence, the cells migrated toward the hillocks to form polypoid nodules (up to 1 mm in diameter) attached to the culture dish by a basal layer of cells. The nodules were composed of several layers of cells and had amorphous, refractile material in their centers. Single cells, removed by gently pipetting a suspension of the nodules in trypsin, excluded trypan blue and could be subcultured in serum or DMB on fibronectin-coated culture dishes. Whole nodules, transferred to fibronectin-coated plates, behaved as tissue explants: cells grew out from the nodules in serum or DMB (Fig. 2). Such nodules have been found in human umbilical vessel smooth muscle cells grown in FBS or calf serum (Fig. 2) (*28*) and in piglet aorta smooth muscle cells grown in FBS (*29*). They are similar to the atherosclerotic plaque-like structures (*29*) seen in human thoracic aorta segments maintained as explant cultures in FBS (*30*).

Table 1. Promotion of the growth of rat abdominal aortic smooth muscle cells in vitro by various hormones. Cells were harvested from stock plates with 0.05 percent trypsin containing 0.2 m*M* EDTA, and a single-cell suspension was made. After treatment with soybean trypsin inhibitor, the cells were centrifuged and resuspended in fresh MEM. Duplicate 35-mm culture wells were coated with fibronectin (10 $\mu g/cm^2$) and inoculated with 10^4 cells in MEM and enough FBS to permit survival without increase in cell number. The cultures were then supplemented with a given hormone and 3 days later were refed. On day 5 the medium was aspirated, 1.0 ml of trypsinated EDTA and 0.2 ml of trypan blue were added to each well, and the number of living cells per well was determined by counting with a hemocytometer. Viability was over 90 percent in all determinations; S.E.M., standard error of the mean.

Hormone	Dose	Mean number of cells ($\times 10^{-4}$) ± S.E.M.
Control		1.5 ± 0.9
PDGF	1 U/ml	11.9 ± 2.6
EGF	10 ng/ml	1.5 ± 0.2
Insulin	100 ng/ml	3.7 ± 0.8
Transferrin	10 μg/ml	15.4 ± 3.2
SM-A*	10 μg/ml	10.5 ± 1.8
SM-C	10 μg/ml	5.9 ± 0.9
MSA	500 ng/ml	6.0 ± 0.4
IGF-I	50 ng/ml	6.7 ± 0.3
Hydrocortisone	$5 \times 10^{-5} M$	2.8 ± 0.5
Alpha-thrombin	1 μg/ml	4.1 ± 1.0
EGF + SM-A	10 ng/ml + 10 μg/ml	15.3 ± 2.2
Insulin + SM-A	100 ng/ml + 10 μg/ml	19.6 ± 0.3

*Somatomedin A; SM-A and SM-C were partially purified from human Cohn fraction IV.

The ability of normal diploid arterial smooth muscle cells to grow and propogate in a serum-free, hormone-supplemented medium extends the theory that the role of serum in cell culture is to provide hormones (*31*). The formation of polypoid nodules, possibly resembling atherosclerotic plaques (*28, 29*), by hormone-supplemented smooth muscle cells is consistent with the concept that serum presents an abnormal environment for cultured smooth muscle cells (*32*), since in vivo they are exposed to the full complement of serum hormones only after endothelial injury (*5, 33*). We believe that we have achieved a more integrated understanding of the influences of hormones on the medial smooth muscle cell (Fig. 3). This may lead to an appreciation of atherosclerosis as a disease mediated by a complex endocrine system.

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Normalization by Cell Fusion of Sister Chromatid Exchange in Bloom Syndrome Lymphocytes

Abstract. *Fusion of fresh lymphocytes from a Bloom syndrome (BS) patient with those of normal subjects or a BS heterozygote resulted in complete normalization of the frequency of sister chromatid exchanges in the chromosomes of BS cells. This normalization took place by the first mitosis in hybrid cells. In contrast, cultivation of BS lymphocytes with those of normal subjects or the BS heterozygote had no effect on sister chromatid exchanges. The cell fusion experiments suggest that the normalization of the sister chromatid exchange frequencies in BS cells can be achieved by factors conserved in the cells of various mammalian species. These findings are compatible with the concept that BS is a recessive genetic mutation at regulatory levels of the DNA repair function.*

Lymphocytes, fibroblasts, and marrow cells of Bloom syndrome (BS) patients exhibit high frequencies of chromosome breaks and rearrangements and spontaneous increases in the number of sister chromatid exchanges (SCE) (*1, 2*). Even though BS cells are competent in excision and postreplication repairs and single-strand break rejoining and have normal levels of DNA polymerases, the findings are compatible with a deficiency in some aspect of the DNA repair function (*3–5*). Bryant *et al.* (*6*) observed normalized SCE frequencies in hybrid cells of BS and euploid cells. Alhadeff *et al.* (*7*) reported normalization of SCE in somatic cell hybrids 15 to 20 days after fusion between Chinese hamster cells (CHO-YH21) and BS fibroblasts (GM 1492) that was independent of the type and number of human chromosomes retained in the hybrid cells. In these studies (*6, 7*), the SCE frequencies were examined 10 to 20 days after fusion and varied from one BS cell to another. The mechanism of normalization of SCE is still obscure, especially in relation to (i) the stage of the cell cycle in hybrids when normalization of the SCE frequencies takes place and (ii) the class of cell populations involved in fusion. Fusion experiments with cells cultured for a long time should be evaluated with caution, since cells with high SCE frequencies were able to correct the defect in BS cells (*8*). Furthermore, cultivation of BS and normal cells led to different results and conflicting conclusions (*9–11*).

In our study, we examined the mechanism of SCE in BS cells by simultaneously performing cocultivation and hybridization experiments, using fresh lymphocytes from normal subjects, a BS heterozygote (HBS), and a BS patient. Fresh lymphocytes were used in order to exclude a possible metabolic shift in hybrids during long-term culture. We report that fusion of BS cells with either normal or HBS cells results in complete correction of the SCE incidence in the BS chromosomes by the first mitosis in hybrid cells, probably occurring during the preceding DNA synthesis period.

Heparinized peripheral blood was collected from two healthy females, a 20-year-old male with BS (*2*), and the mother of another BS patient, (HBS). The lymphocytes were purified by the Ficoll-Hypaque density sedimentation method (*12*).

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