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Inhibitory effects of suramin on androgen-dependent and -independent growth of neonatal mouse seminal vesicles in vitro

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Abstract The effects of suramin on the growth of seminal vesicles (SVs) of neonatal mice were investigated in vitro. SVs from 0-day-old male mice were cultured in serum-free chemically defined medium supplemented with 5α-dihydrotestosterone (DHT, 10^{-8} M) and insulin (10 μg/ml), alone and in combination. Prior to culture, SVs from 0-day-old mice had no epithelial branches. SVs cultured in medium with DHT formed numerous epithelial branches, while epithelial branching did not occur in SVs cultured without DHT. The addition of suramin (0.2 mM) to medium containing DHT inhibited the formation of epithelial branches almost completely. Removal of suramin from the medium on days 2, 4, and 6 of culture initiated the formation of epithelial branches. Suramin (0.2 mM) reversibly decreased ^{3}H-thymidine-labeling indices (^{3}H-LI) of both epithelium and mesenchyme of SVs cultured in medium with DHT plus insulin or DHT alone during 8 days of culture. Suramin also decreased ^{3}H-LI of both epithelium and mesenchyme of SVs cultured in medium with insulin alone. The present study indicates that suramin reversibly inhibits not only androgen-dependent but also androgen-independent growth and ductal branching morphogenesis of neonatal mouse SVs.

Key words Seminal vesicle · Suramin · Androgen · Insulin · Organ culture · Epithelial growth

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Androgen-dependent development of the seminal vesicles (SVs) and prostate occurs via mesenchymal-epithelial interactions [6]. Paracrine signals from mesenchyme may involve growth factors or extracellular matrix molecules. Two likely growth factor candidates are hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF). Suramin is a polysulfonated naphthylurea that has been used to treat trypanosomiasis and onchoceriasis [15]. Because of its antitumor effects, suramin has been used for treatment of a variety of neoplasms. Although various intracellular actions of suramin have been reported, it has been shown to inhibit the binding of a variety of growth factors to their cell surface receptors. Previous studies have shown that neonatal mouse SVs develop normally in a chemically defined serum-free medium in the presence of androgen, providing a useful model to investigate possible involvement of paracrine growth factors in androgen-dependent mesenchymal signal transmission [14, 17]. Therefore, in the present study we examined the effects of suramin on androgen-dependent and -independent growth and ductal morphogenesis of neonatal mouse SVs.

Materials and methods

Mice and tissues

BALB/c mice 2–3 months old were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Female and male mice were kept together in cages, and, when pregnancy was detected, pregnant mice were housed separately in individual cages. The birth of offspring was examined at noon every day. SVs were removed from 0-day-old (day of birth) male mice and used for organ culture.

Media and organ culture

SVs were cultured in the manner described previously [14, 17]. Briefly, six or seven SVs from 0-day-old mice were placed on

a Millipore filter (Millipore, Bedford, Mass., USA) supported on triangular stainless steel grids inside 35-mm petri dishes (Falcon, Oxnard, Calif., USA). Approximately 2 ml of the serum-free medium was added to the dishes to bring the filter into contact with the medium, and the dishes were placed on a rocker platform in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air. The tissues were cultured for 2, 4, 6, or 8 days. The medium was changed every 2 days.

The serum-free medium consisted of Dulbecco's Modified Eagle's Medium-Ham's F-12 medium (1:1, vol/vol) supplemented with human transferrin (10 μg/ml; Wako Chemicals, Osaka, Japan) and bovine serum albumin (BSA) (5 mg/ml; Sigma, St. Louis, Mo., USA). Powdered medium was obtained from Gibco Laboratories (Grand Island, N.Y., USA). SVs were grown in the serum-free medium with 5α-dihydrotestosterone (DHT; Fluka Chemika, Buchs, Switzerland) at 10^{-8} M alone, insulin (Sigma) at 10 μg/ml alone, or in combination in the presence or absence of suramin at the concentrations indicated. Suramin was supplied by Bayer AG (Wuppertal, FRG).

Estimation of branching morphogenesis

Branching of epithelium was evaluated by counting epithelial branches in photographs of SV whole mounts.

Protein and DNA assays

SVs were homogenized in 100 μl distilled water by sonication, and the protein content was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif., USA). The DNA content was assayed by a modified method of Fiszer-Szafarz et al. [7]. For DNA assay, four SVs were homogenized in 200 μl of 1 N NH_4OH containing 0.2% Triton X-100 by sonication, and dissolved by incubating the homogenate at 90 °C for 2 h. Fifty milliliters of the homogenate was neutralized by the addition of 12.5 ml 4 N HCl and incubated at 60 °C for 30 min after the addition of the 3,5-diaminobenzoic acid solution (300 mg/ml 4 N HCl). After the incubation, the solution was mixed with 2 ml 1 N HCl, and the fluorescence was measured with a Hitachi fluorescent spectrophotometer F-3000 (Hitachi, Tokyo, Japan); excitation and emission were 408 and 500 nm, respectively.

Autoradiographic analysis

Cellular proliferation in cultured SVs was assayed by [^{3}H]thymidine autoradiography. SVs cultured for 2, 4, 6, or 8 days were incubated in 2 ml Dulbecco's Modified Eagle's Medium containing 5 μCi/ml [methyl-^{3}H]-thymidine (80 Ci/mmol, New England Nuclear, Boston, Mass., USA) for 5 h at 37 °C in 5% CO_2 and 95% air. SVs were then fixed in phosphate buffered (0.1 M, pH 7.4) 10% formalin, dehydrated, embedded in paraffin and serially sectioned at 2 μm. The sections were mounted on glass slides, deparaffinized, and dipped in photographic emulsion (NR-M2, Konica Industries, Tokyo, Japan). After exposure for 21 days, the autoradiograms were developed and stained with hematoxylin and eosin. The [^{3}H]-thymidine labeling index (^{3}H-LI) was obtained by counting at least 1000 epithelial or mesenchymal cells per SV.

Statistical analysis

Statistical significance ($P < 0.05$) was determined by Student's *t*-test.

Results

Effects of suramin on branching morphogenesis SVs

Figure 1 shows representative whole-mount photographs of SVs from 0-day-old mice before culture and after 6 days of culture in medium with DHT and insulin, DHT alone, and insulin alone in the presence or absence of suramin (0.2 mM). SVs from 0-day-old mice before culture consisted of a simple epithelial tube

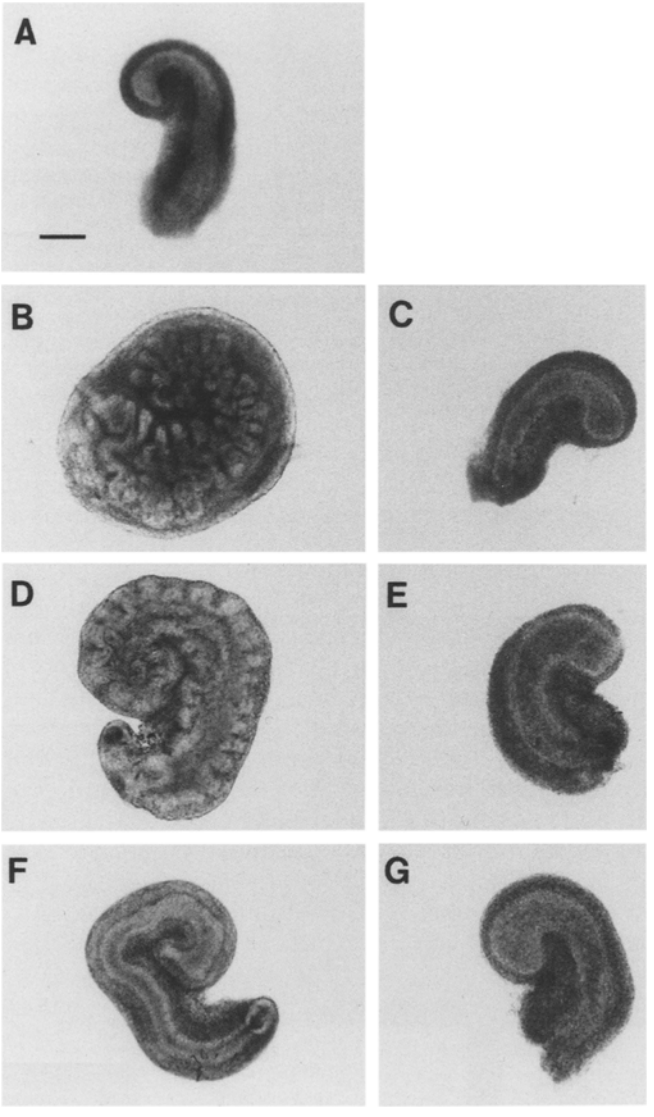

Fig. 1A–G Representative whole-mount pictures of SVs. **A** SV from a 0-day-old (day of birth) mouse before culture. **B** SV cultured in medium with DHT (10^{-8} M) plus insulin (10 μg/ml) for 6 days. **C** SV cultured in medium containing DHT plus insulin in the presence of suramin (0.2 mM) for 6 days. **D** SV cultured in medium with DHT for 6 days. **E** SV cultured in medium with DHT in the presence of suramin (0.2 mM) for 6 days. **F** SV cultured in medium with insulin for 6 days. **G** SV cultured in medium with insulin in the presence of suramin (0.2 mM) for 6 days. *Bar* in **A** represents 200 μm

surrounded by mesenchyme (Fig. 1A). SVs grown in medium with DHT plus insulin increased in size, and the epithelium showed extensive branching (Fig. 1B). Suramin almost completely inhibited androgen-induced epithelial branching as well as the increase in size (Fig. 1C). SVs grown with DHT alone exhibited modest growth and epithelial branching (Fig. 1D). Addition of suramin to the medium with DHT alone inhibited the growth and epithelial branching (Fig. 1E). SVs cultured in medium with insulin alone increased in size and were devoid of epithelial branching (Fig. 1F). SVs cultured in medium with insulin alone in the presence of suramin were also devoid of epithelial branching (Fig. 1G).

Effects of suramin on SVs cultured in medium with DHT plus insulin

The time course of epithelial branching in SVs cultured in medium with DHT plus insulin is shown in Fig. 2. Epithelial branching was evaluated as number of branches per SV. In culture with DHT plus insulin, the number of branches increased sharply after 2 days and gradually after 4 days of culture. The average number of branches was 33.9 per SV on day 8 of culture. Suramin (0.2 mM) almost completely inhibited epithelial branching. Removal of suramin from the medium on days 2, 4, and 6 of culture resulted in an abrupt increase in the number of epithelial branches although the number of branches on day 8 of culture was less than that of SVs cultured in medium without suramin

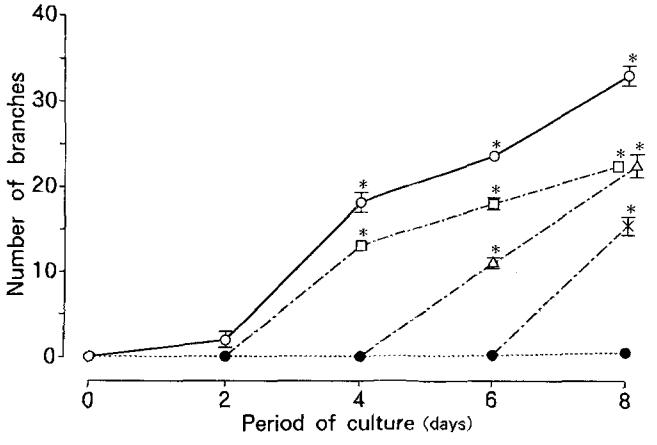

Fig. 2 Time course effects of suramin on number of epithelial branches of SVs cultured in medium with DHT plus insulin. SVs from 0-day-old mice were cultured in medium with DHT plus insulin in the presence (●–●), or absence (○–○) of suramin (0.2 mM). To assess recovery from the effects of suramin, SVs were also cultured for 2 days in medium with DHT, insulin and suramin (0.2 mM), and subsequently in medium with DHT plus insulin but without suramin after 2 days (□–□), 4 days (△–△), and 6 days (×–×) of culture. *Each point* represents the mean ± SE of five to thirteen SVs. *$P < 0.05$, significant difference from the value for SVs cultured in medium with suramin

for the entire culture period. Numbers (means ± SE) of epithelial branches on day 6 of culture with DHT plus insulin in the absence of suramin, and in the presence of suramin at 0.02, 0.05, 0.1, and 0.2 mM were 25.6 ± 1.2 ($n = 7$), 25.6 ± 0.8 ($n = 7$), 25.7 ± 0.4 ($n = 7$), 13.3 ± 0.8 ($n = 13$), 0.15 ± 0.15 ($n = 13$) per SV, respectively, and suramin at concentrations more than 0.1 mM significantly reduced the number of epithelial branches ($P < 0.05$) in SVs grown in medium supplemented with DHT plus insulin.

The culture medium also contained transferrin at 10 μg/ml. Deletion or enrichment of the concentration of transferrin to 20 times that of our standard medium (200 μg/ml) did not affect the number of epithelial branches induced by insulin and DHT; numbers of epithelial branches (means ± SE) at 0, 10, and 200 μg/ml transferrin on day 6 of culture were 23.1 ± 1.0 ($n = 8$), 25.6 ± 1.2 ($n = 7$), and 21.3 ± 0.8 ($n = 6$), respectively. Suramin at 0.2 mM also completely inhibited epithelial branching morphogenesis at all transferrin concentrations (0–200 μg/ml).

Figure 3 shows effects of suramin on ^{3}H-LI of epithelium and mesenchyme of SVs cultured in medium with DHT plus insulin. The ^{3}H-LI of epithelium of SVs from 0-day-old mice, which was 4.3% at the beginning of culture, increased about seven-fold on day 2 of culture and decreased gradually thereafter. Suramin (0.2 mM) markedly suppressed epithelial ^{3}H-LI. Removal of suramin on days 2, 4, and 6 of culture allowed epithelial ^{3}H-LI to rebound to levels comparable to that of SVs cultured in medium without suramin. ^{3}H-LI of mesenchyme of SVs increased about two-fold on day 2 of culture and decreased gradually as was the case for the epithelium. Suramin markedly decreased mesenchymal ^{3}H-LI during 8 days of culture. Removal of suramin from the medium on days 2, 4, and 6 of culture increased the ^{3}H-LI in the mesenchyme to levels comparable to that of SVs cultured in medium without suramin. When the effects of suramin at various concentrations were examined on day 6 of culture (Fig. 4), suramin at concentrations more than 0.1 mM significantly decreased ^{2}H-LI of both epithelium and mesenchyme.

The protein content of SVs at the beginning of the culture was 6.8 ± 0.3 μg/SV (mean ± SE, $n = 7$), and those (means ± SE, μg/SV) on days 2, 4, 6, and 8 in culture with DHT plus insulin in the presence vs. absence of suramin (0.2 mM) were 10.1 ± 0.4 ($n = 7$) vs. 12.9 ± 0.4 ($n = 7$), 8.9 ± 0.4 ($n = 7$) vs. 16.4 ± 0.9 ($n = 7$), 9.3 ± 0.5 ($n = 6$) vs. 23.4 ± 0.5 ($n = 7$), and 11.7 ± 1.1 ($n = 5$) vs. 25.9 ± 1.4 ($n = 7$), respectively. Differences were significant on days 2, 4, 6, and 8 ($P < 0.05$).

The DNA content (mean ± SE) of SVs was 0.61 ± 0.01 μg/SV ($n = 4$) at the beginning of the culture. The DNA contents (means ± SE) of SVs cultured in medium with DHT plus insulin in the presence and absence of suramin (0.2 mM) on day 6 of culture were

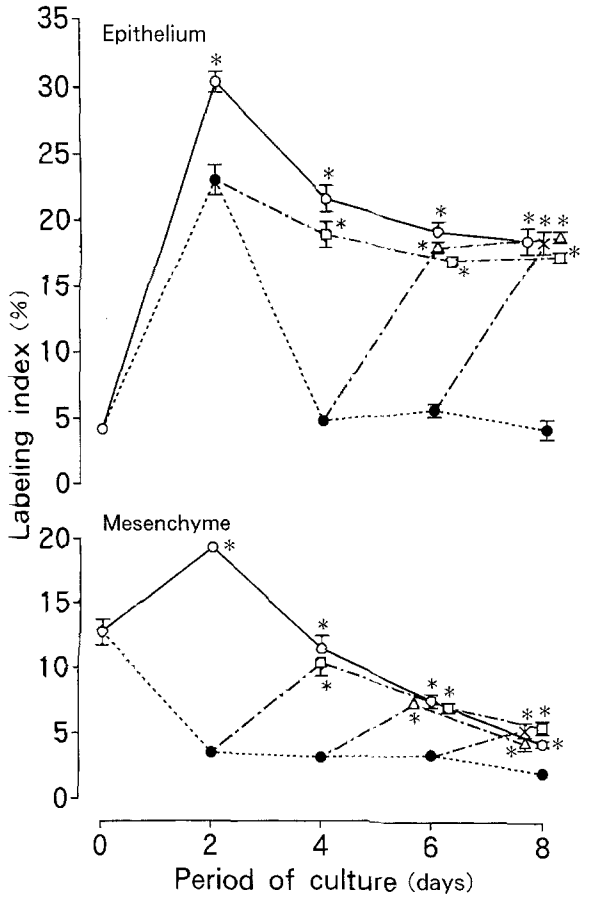

Fig. 3 Effects of suramin on labeling indices of epithelium and mesenchyme of SVs cultured in medium with insulin plus DHT. SVs from 0-day-old mice were cultured in medium with insulin plus DHT in the presence (●–●) or absence (○–○) of suramin (0.2 mM). To assess recovery from the effects of suramin, SVs were also cultured for 2 days in medium with DHT, insulin and suramin (0.2 mM), and subsequently in medium with DHT plus insulin but without suramin after 2 days (□–□), 4 days (△–△) and 6 days (×–×) of culture. *Each point* represents the mean ± S.E. of five to ten SVs. $*P < 0.05$, significant difference from the value for SVs cultured in medium with suramin

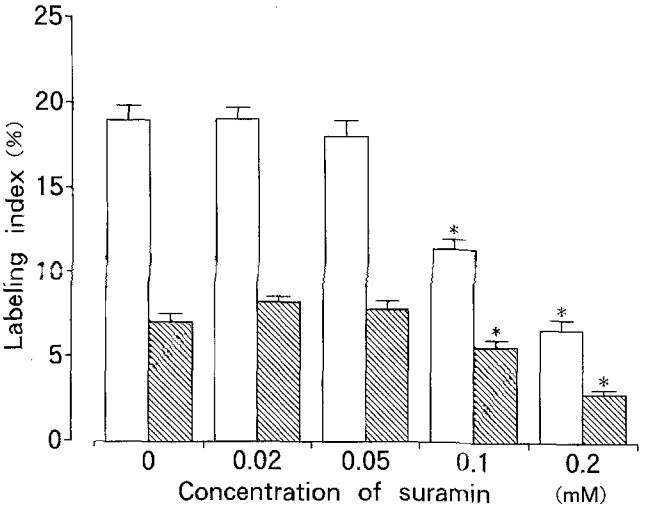

Fig. 4 Effects of suramin at various concentrations on labeling indices of epithelium (□) and mesenchyme (▨) of SVs cultured in medium with insulin plus DHT. SVs from 0-day-old mice were cultured in medium with insulin plus DHT in the presence of suramin at various concentrations for 6 days. *Each bar* indicates the mean + S.E. of four to eight SVs. $*P < 0.05$, significant difference from the value for SVs cultured in medium without suramin

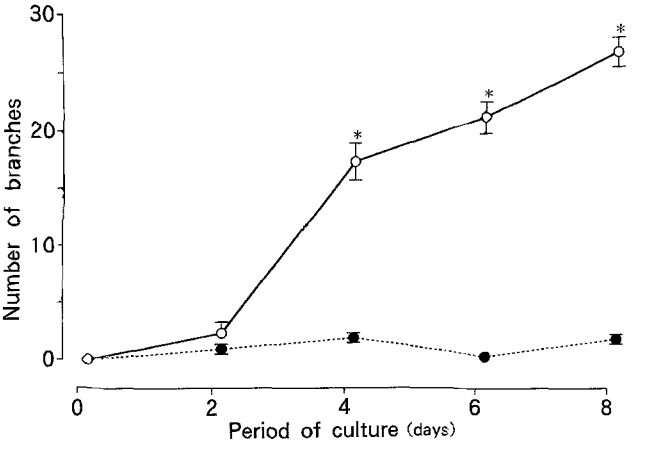

Fig. 5 Time course effects of suramin on the number of epithelial branches of SVs cultured in medium with DHT alone in the presence (●–●) or absence (○–○) of suramin (0.2 mM). *Each point* indicates the mean ± SE of five to seven SVs. $*P < 0.05$, significant difference from the value of SVs cultured in medium with suramin

1.04 ± 0.03 ($n = 8$), and 2.12 ± 0.08 ($n = 4$) μg/SV, respectively. These values were significantly different ($P < 0.05$).

Effects of suramin on SVs cultured in medium with DHT alone

The time course of epithelial branching in SVs cultured in medium with DHT alone is shown in Fig. 5. In culture with DHT alone the number of branches increased sharply after 2 days of culture, and gradually after 4 days. The average number of branches was 27.9 per SV on day 8 of culture. Suramin (0.2 mM) almost completely inhibited epithelial branching.

Figure 6 shows the effects of suramin on ^{3}H-LI of epithelium and mesenchyme of SVs cultured in medium with DHT alone. The labeling index of epithelium of SVs from 0-day-old mice increased about five-fold on day 2 of culture and decreased gradually thereafter. Suramin (0.2 mM) markedly decreased the epithelial ^{3}H-LI at all time points. The ^{3}H-LI of mesenchyme of SVs decreased gradually after 2 days in culture with DHT alone. Suramin markedly decreased the mesenchymal ^{3}H-LI on days 2, 4, and 6 of culture.

The protein contents (means ± SE, μg/SV) on days 2, 4, 6, and 8 in culture with DHT alone in the presence vs. absence of suramin (0.2 mM) were 5.6 ± 0.7 ($n = 9$)

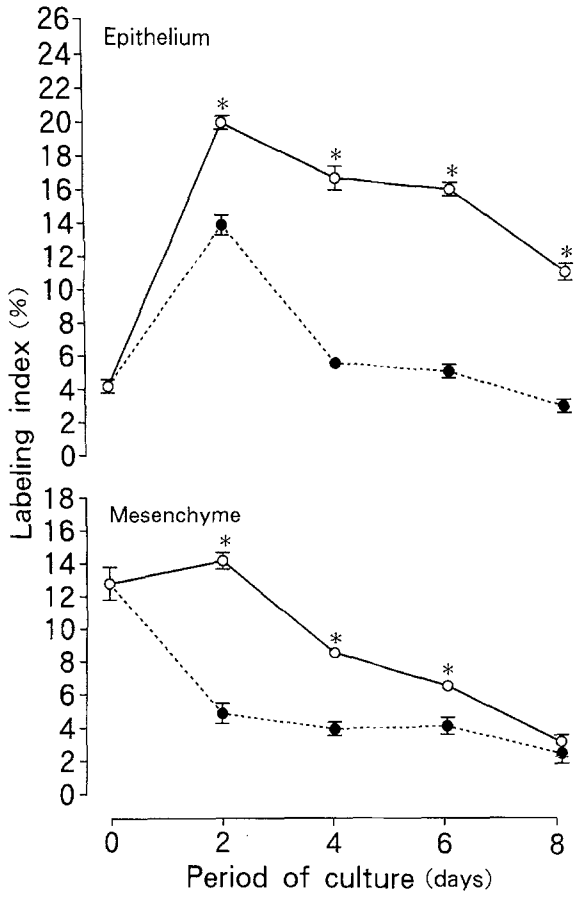

Fig. 6 Time course effects of suramin on labeling indices of epithelium and mesenchyme of SVs cultured in medium with DHT alone in the presence (●–●) or absence (○–○) of suramin (0.2 mM). *Each point* indicates the mean ± SE of five to seven SVs. *$P < 0.05$, significant difference from the value of SVs cultured in medium with suramin

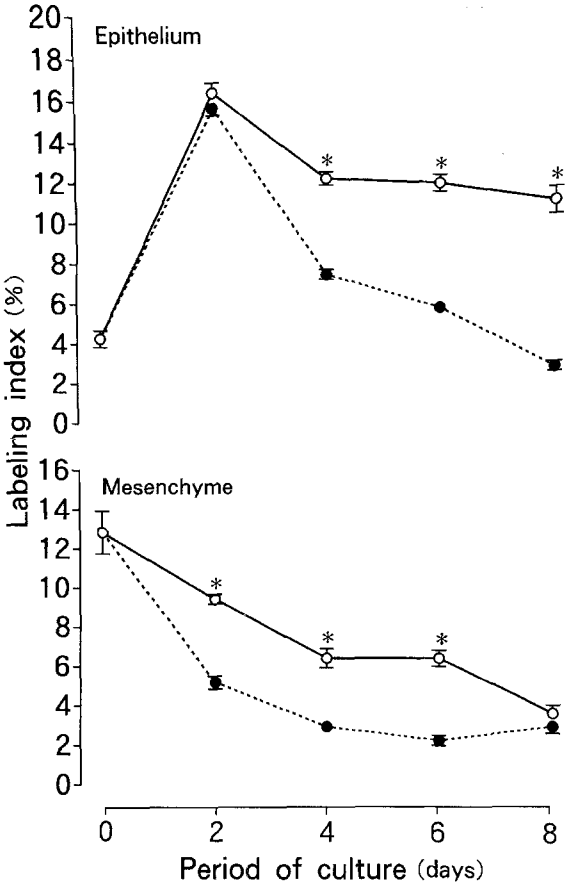

Fig. 7 Time course effects of suramin on labeling indices of epithelium and mesenchyme of SVs cultured in medium with insulin alone in the presence (●–●) or absence (○–○) of suramin (0.2 mM). Each point indicates the mean ± SE of five to seven SVs. *$P < 0.05$, significant difference from the value of SVs cultured in medium with suramin

vs. 5.2 ± 0.7 ($n = 6$), 7.9 ± 0.7 ($n = 6$) vs. 12.1 ± 1.4 ($n = 6$), 6.2 ± 0.2 ($n = 5$) vs. 14.7 ± 1.7 ($n = 5$), and 6.8 ± 0.3 ($n = 7$) vs. 23.8 ± 1.4 ($n = 6$), respectively. Differences were significant on days 4, 6, and 8.

The DNA content of SVs at the beginning of the culture was 0.61 ± 0.01 μg/SV (mean ± SE, $n = 4$). The DNA contents (means ± SE) were 1.86 ± 0.11 ($n = 5$) and 1.16 ± 0.05 ($n = 5$) μg/SV on day 6 of culture in medium without suramin and with suramin (0.2 mM), respectively. These values were significantly different ($P < 0.05$).

Effects of suramin on SVs cultured in medium with insulin alone

Figure 7 shows the effects of suramin on ^{3}H-LI of epithelium and mesenchyme of SVs cultured in medium with insulin alone. The ^{3}H-LI of epithelium of SVs from 0-day-old mice increased about three-fold on day 2 of culture, and decreased slightly thereafter. Suramin (0.2 mM) decreased epithelial ^{3}H-LI after 2 days of culture. ^{3}H-LI of mesenchyme of SVs cultured with insulin alone decreased gradually during the culture. Suramin significantly decreased the ^{3}H-LI on days 2, 4, and 6 of culture.

The protein contents (means ± SE, μg/SV) of SVs on days 2, 4, 6, and 8 in the presence vs. absence of suramin (0.2 mM) were 8.8 ± 0.4 ($n = 7$) vs. 9.7 ± 0.3 ($n = 7$), 12.6 ± 0.6 ($n = 6$) vs. 15.6 ± 0.4 ($n = 7$), 10.2 ± 0.2 ($n = 7$) vs. 15.4 ± 0.6 ($n = 7$), and 9.8 ± 0.5 ($n = 6$) vs. 21.0 ± 1.1 ($n = 7$), respectively. Differences were significant on days 4, 6, and 8 ($P < 0.05$).

The DNA content (mean ± SE) of SVs at the beginning of the culture was 0.61 ± 0.01 μg/SV($n = 4$). The DNA contents (means ± SE) of SVs cultured in medium with insulin alone in the presence and absence of suramin at 0.2 mM on day 6 of culture became 1.15 ± 0.03 ($n = 5$) and 1.54 ± 0.05 ($n = 5$) μg/SV, respectively. These values were significantly different ($P < 0.05$).

Discussion

The organ culture method used in the present study constitutes a refinement of the system employed previously [1, 14, 16, 17]. In all of these studies including the current one, androgens can elicit epithelial growth and branching morphogenesis in the developing SV, as well as an increase in DNA and protein contents. Because of the simplicity of the medium, it is possible to determine the effects of single agents such as suramin.

The medium used in the present study contains transferrin at 10 μg/ml. Forsbeck et al. [8] reported that suramin suppressed the binding of transferrin to its cell surface receptors. However, in our culture system, the concentration of transferrin did not affect the inhibitory action of suramin. Therefore the inhibitory actions of suramin found in the present study are not due to perturbation of the interaction of transferrin to its receptor.

Our results showed that the effects of suramin were reversible. Suramin inhibited androgen-induced growth of neonatal SVs and androgen-induced epithelial morphogenesis. In SVs of mice at birth, androgen receptors (ARs) are detectable only in mesenchyme, whereas epithelial ARs are detectable on postnatal day 1 or 2 and are maintained thereafter [4]. In organ culture of 0-day-old SVs in serum-free medium, epithelial ARs have also been detected at 3 days of culture [14]. Thus, in organ cultures of SVs, androgens may be acting directly on the epithelium mediated by intraepithelial ARs or indirectly via paracrine mechanisms mediated by mesenchymal ARs. It should be recognized that the mere expression of epithelial ARs does not imply that a given biological effect of androgen is elicited by epithelial AR. Through tissue recombinant experiments using Tfm (testicular feminization) tissue, it is evident that androgen-dependent ductal morphogenesis, epithelial proliferation, and certain aspects of epithelial differentiation are elicited via paracrine effects from mesenchyme [5, 6]. By contrast, expression of androgen-dependent secretory proteins is dependent on epithelial ARs [5].

Suramin inhibits the binding of a variety of growth factors to their receptors [2, 3, 9–12, 19]. This suggests suramin may be inhibiting androgen-dependent and androgen-independent autocrine/paracrine interactions.

Suramin also inhibited insulin-induced growth of SVs. Insulin binds to insulin receptor and insulin-like growth factor I receptor [13]. At present it is not known which receptor is essential for insulin-induced growth of SVs. Inhibitory actions of suramin on insulin-induced growth may be due to its effects on the interaction of insulin and receptors and/or its intracellular effects including inhibition of some enzyme activities [9, 18].

In the present study, suramin inhibited not only androgen-dependent growth of SVs, but also androgen-independent growth and ductal branching morphogenesis. It is likely that suramin elicits its inhibitory effects via interference with growth factor/receptor interactions. The importance of our SV organ culture system in assessing the role of trophic as well as inhibitory agents such as suramin is that the SV organ culture system is multidimensional, providing information relevant to both growth and complex ductal branching morphogenesis. It should also be stressed that the growth and development observed in SVs in vitro closely reflect in vivo events. Thus, the organ culture of SVs provides a useful model for studying the mechanisms of androgen-dependent and -independent growth and development of the male reproductive organs.

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