VC Dhulipala
WV Welshons
CS Reddy

Inhibition of human embryonic palatal mesenchymal cell cycle by secalonic acid D: a probable mechanism of its cleft palate induction

Authors' affiliations:
V.C. Dhulipala, W.V. Welshons, C.S. Reddy,
Department of Biomedical Sciences,
University of Missouri, Columbia, MO, USA

Correspondence to:
Chada S. Reddy
Department of Veterinary Biomedical Sciences
University of Missouri
1600 E Rollins St
Columbia, MO 65211, USA
Tel.: +1 573 882 7653
Fax: +1 573 884 6890
E-mail: reddyc@missouri.edu

Dates:
Accepted 21 May 2004

To cite this article:
Orthod Craniofacial Res **7**, 2004; 227–236
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Structured Abstract

Authors – Dhulipala VC, Welshons WV, Reddy CS

Objective – To assess the mechanism(s) of cleft palate induction by secalonic acid D (SAD) in human embryonic palatal mesenchymal (HEPM) cells and compare them with those evaluated in the murine embryonic palate.

Design – Effect of SAD on HEPM cell proliferation was studied by obtaining dose response curves for cell numbers, uptake of ^{3}H-thymidine and the expression of proliferating cell nuclear antigen (PCNA). Effects of SAD on cell cycle were assessed by flowcytometry. Cell-labeling with ^{3}H-glucosamine and immunoblot analysis were conducted to study SAD effects on the synthesis of glycosaminogycans (GAG) and the expression of fibronectin and tenascin, respectively.

Results – SAD induced a concentration-dependent decrease in HEPM cell number and ^{3}H-thymidine uptake beginning at 0.1 μg of SAD/ml. Expression of PCNA and progression of cell cycle from G1 to S phase were inhibited following SAD exposure. Cell viability was significantly reduced only at 7.5 μg/ml of SAD or higher indicating that the reduction in cell numbers by SAD at lower concentrations is likely due to reduced proliferation and at higher concentrations due to both reduced proliferation and cell death. Synthesis of extra cellular matrix components (GAGs, fibronectin or tenascin) by HEPM cells, however, was not inhibited by SAD.

Conclusion – The results of these studies confirmed those of our previous studies with mice and the MEPM cells that SAD may induce cleft palate by reducing numbers of palatal mesenchymal cells by inhibition of their proliferation thereby leading to a reduction in the size of the developing palate shelves.

Key words: cleft palate; extra cellular matrix; human embryonic palatal mesenchyme; proliferation; secalonic acid D

Introduction

Cleft palate is a common birth defect in human beings world-wide, occurring at a rate of one in 700 newborns in the US, and this rate is increasing (1,2). A failure of the bilateral palatal shelves to elevate and make contact with each other or failure of the apposing shelves to fuse can lead to cleft palate. Failure of elevation can result from smaller shelves or altered osmotic forces within the shelves. Smaller shelves can result from a reduction in the number of embryonic palatal mesenchymal cells (which form the bulk of the palate) or because of a reduction in the extra cellular matrix (ECM). The latter also adds to the osmotic pressure within the shelves thought to contribute to their elevation. Although the exact mechanism of fusion is unknown, prevailing hypotheses invoke either apoptosis or dedifferentiation of the medial edge epithelial cells as leading to the disappearance of the epithelial seam separating the two apposing shelves. A number of environmental contaminants and chemicals are known to produce cleft palate in animals by one or more of the mechanisms mentioned above.

Secalonic acid D (SAD) is a mycotoxin produced in corn by *Pencillium oxalicum* as a common contaminant in the Unites states of America (3). Although its effects in humans have not been studied, data form the mouse model suggests that as little as 5 mg SAD/kg body weight, intraperitoneally, can cause 7% incidence in the offspring (4). Adjustment of this dose for 11-fold lower sensitivity by oral route in mice (5) and application of a safety factor of 10 as is done routinely in risk assessment will yield a minimal teratogenic dose of 5.5 mg/kg or less in humans. Using per capita corn consumption of 45 lb/year in humans (World Book Encyclopedia, 1981) and the presence of up to 5 g of SAD/kg of contaminated corn in the laboratory (6), dietary exposure to SAD in an average pregnant woman can reach 250 mg/day. In addition, pregnant women in grain processing industries can be exposed to up to 100 mg/day from inhalation exposure to grain dust containing up to 25 mg SAD/kg (7) and a minute respiratory volume of 6 l/min of air containing dust at 250 mg/m^3 over a workday of 8 h (8). This translates into an exposure rate of 7.5 mg of SAD/kg body weight for an average woman weighing 55 kg. This analysis suggests a clear danger from SAD exposure to the offspring of exposed women.

The cleft palate induced by SAD in the mouse offspring has been shown to be a result of smaller palatal shelves that fail to elevate (9). In support of this mechanism, SAD has been shown to inhibit mouse embryonic palatal mesenchymal (MEPM) cell proliferation without affecting apoptosis or ECM synthesis (10). At the molecular level, SAD affected the mouse palatal signaling pathways involving protein kinases A (PKA) and C (PKC) at multiple sites, leading to a reduction in the binding of transcription factors to the respective DNA response elements and ultimately leading to reduced expression of cell cycle genes such as that of proliferating cell nuclear antigen (PCNA) (11, 12). The human relevance of these findings, however, is unknown. The human embryonic palatal mesenchymal (HEPM) cell line derived from the secondary palate of a human abortus during the time of palatal shelf closure with a female diploid karyotype served as a starting point in these investigations. With plating efficiencies of up to 95%, notable display of contact growth inhibition following an exponential growth phase that lasted for approximately 6 days, a total cell cycle transit time of approximately 22 h, and no dramatic change in chromosomal complement between passage 5 and 14, these cells were judged to have met the criteria regarding karyotype stability that were assumed suitable for testing by the National Toxicology Program of the USA (13). Despite the expected differences in responses compared to those in MEPM cells (14), the advantages of using the HEPM cell line in these studies relate not only to bridging the

species differences between the mouse and the human being but also to allow assessment of the relevance of mechanism of action deemed to be significant based on animal studies. Use of this cell type will also allow identification of other agents inducing CP in humans by altering HEPM cell function and, because of their stability, allow functional molecular biological studies (such as stable transfection of genes of interest) to assess the relevance of target biomolecules identified in animal studies.

To this end, the following studies were conducted to test the hypothesis that SAD inhibits the proliferation/cell cycle of, but not ECM synthesis by, the HEPM cells in culture in a fashion similar to that seen in MEPM cells.

Materials and methods

Materials

Secalonic acid D was extracted and purified as described by Reddy et al. (1979). Opti-minimum essential medium (Opti-MEM) and other tissue culture materials were purchased from Gibco-BRL (Life Technologies, Gaithersburg, MD and Irvine sci, CA, USA). [^{3}H]thymidine (20 Ci/mmol) and [^{3}H]glucosamine (21.6 Ci/mmol) were obtained from NEN Biolabs (Boston, MA, USA). Monoclonal anti-fibronectin antibody, cetylpyridinium chloride (CPC), papain, purified hyaluronic acid (HA) and chondroitin sulfate (CS), proteinase K, RNase A, hyaluronidase and other routine chemicals were from Sigma Chemicals (St Louis, MO, USA). Polyclonal anti-PCNA and anti-Tenascin antibodies were from Santa Cruz Biotech (Santa Cruz, CA, USA).

Cell culture and treatment

Human embryonic Palatal mesenchymal cell line was obtained from American type cell culture (ATCC) bank at 7th passage level and cultures were established. The cell line was expanded and aliquots of cells were frozen for further use. All the experiments were conducted with cells in passages between 8 and 13. Cells were plated into 35 mm tissue culture dishes (6 well plates) at a density of 2.5×10^4 cells/well in O-MEM containing 2.5% human serum (AB type), 2 mM glutamine, 55 μM mercaptoethanol, and antibiotic-antimycotic solution (150 U penicillin; 150 μg/ml streptomycin; 0.37 μg/ml amphotericin B). The cultures were incubated at 37°C in a humidified atmosphere and 5% CO_2 with media changed every day till the time of treatment. The cells were exposed to SAD (final concentration of 0.025, 0.1, 0.45, 1.9, 7.5 and 30 μg/ml) or vehicle (DMSO at a final concentration of 0.1% in the medium) once the cultures attained ~40% confluence (~72 h). Following 48 h of incubation, the media were collected, the cell sheet was washed with PBS, dissociated from the plates by incubating with 0.05% trypsin and 0.5% EDTA, neutralized with the medium, pelleted and preserved at −80°C for further use.

Measurement of DNA synthesis

This was performed as described by Hanumegowda et al. (10) except that the control and SAD treated cells were pulse-labeled with [^{3}H]thymidine (2 μCi/ml) for 4 h before the completion of the 48-h SAD/vehicle exposure period. Thymidine incorporation was measured as trichloroacetic acid (TCA)-precipitable radioactivity as described previously. Briefly, the harvested cells were washed twice with ice-cold CMF-PBS, then three times with ice-cold 5% TCA and finally two times with absolute ethanol. The contents were then solubilized in 10 mM EDTA pH 12.3 at 37°C for 20 min and neutralized to pH 7.2 with 0.77 M KH_2PO_4. Aliquots were drawn for measurement of radioactivity and estimation of protein content. Concentration of protein was estimated by the method of Bradford (15). Radioactivity was measured by liquid scintillation counting and the counts were expressed as dpm per unit of protein.

Immunoblots for PCNA in the nucleus

The cells were cultured and treated (with IC_{50}) as above and the cells obtained from these groups were fractionated into cytoplasmic and nuclear extracts according to Hanumegowda (12). Briefly, the cells were first lysed with a low salt buffer containing 1.5 mM $MgCl_2$,10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 mM PMSF, 10 μg/ml leupeptin and 1 μg/ml of aprotinin in 10 mM HEPES, pH 7.9 on ice for 10 min and then homogenized by vortexing them for 15 s. The samples were centrifuged and the supernatant collected is the cytoplasmic extract and the pellet was re-suspended into a high salt solution containing 1.5 mM $MgCl_2$,420 mM NaCl, 10 μM NaF, 0.5 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml

of aprotinin and 0.2 mM EDTA in 20 mM HEPES, pH 7.9 on ice for 15 min with constant vortexing and then micro centrifuged. The supernatants (Nuclear extracts) were used for the immunoblot analysis of PCNA. Five micrograms of nuclear extract was electrophoreses on a 4–15% denaturing gel and transferred on to a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk, washed with PBS-Tween, probed with anti-PCNA (1:1000) antibody and reacted with horse radish peroxidase-conjugated anti-mouse (1:1500) (Santa Cruz Biotechnology, Santa Cruz, CA and Calbiochem, La Jolla, CA, USA). The bands were then visualized by using enhanced chemiluminesence (NEN Life Science, Boston, MA, USA) and quantitated by densitometry.

Estimation of cell number and viability

This was performed according to Hanumegowda et al. (10). The standard counting technique using hemocytometer was used to estimate the total cell number and density. Cell viability determination was done by the trypan blue dye-exclusion technique. Briefly, 0.4% solution of trypan blue (to a final concentration of 0.2%) was added to a known volume of aliquot of the harvested cells and the percent of cells containing stained and unstained nuclei were estimated by counting in a hemocytometer.

Measurement of ECM synthesis

Incorporation of [^{3}H]glucosamine into glycosaminogycan (GAG) and selective fractionation techniques (16) were used to measure sGAG and HA synthesis. Briefly, the cells were metabolically labeled with [^{3}H]glucosamine (3 μCi/ml) for 24 h along with a 48-h exposure to SAD or vehicle. The culture medium was collected and aliquots of 0.5 ml were proteolysed with 0.5% papain in the presence of 5 mM EDTA and 5 mM cysteine-HCl at 60°C for 6 h. The samples were then boiled and precipitation of GAG was accomplished with addition and incubation with 2% CPC in 20 mM NaCl for 3 h at room temperature. The precipitate was then collected on a 0.45 μm mixed cellulose ester filtration membrane (Millipore, Bedford, MA, USA) by repeated washings with 1% CPC until no more radioactivity was detected in the filtrate. Radioactivity on the membrane containing the precipitate represented the total precipitable labeled GAG and the HA portion of the precipitate was solubilized with 0.5 N HCl and washed off the membrane with 1% CPC solution and thus fractionated from the sGAGs portion of the precipitate and the amounts of each of these components were assessed in a scintillation counter (Ls 60001C; Beckman Coulter, Inc., Fullerton, CA, USA) and extrapolated as the amount of GAG synthesized/million cells (taking the number of cells in each group at the time of treatment).

Immunoblots for fibronectin and tenascin

Extracts from cellular portion were prepared by using a buffer containing 0.1 mM EDTA and 1% triton X-100 with protease inhibitors in 30 mM tris-HCl (pH, 7.4). Equal quantities of either the media (100 μg protein) or the cellular fractions (30 μg protein) for analysis of fibronectin and tenascin, were separately electrophoresed on a 6% polyacrylamide gel with SDS and then transferred onto nitrocellulose membrane overnight at 4°C. The membranes were blocked with 5% non-fat dry milk, washed with PBS-Tween, probed with anti-fibronectin (1:500) or anti-tenascin (1:200) antibodies and reacted with horse radish peroxidase-conjugated anti-mouse or anti-goat antibodies (1:500 for tenascin and 1:1000 for fibronectin) (Santa Cruz Biotechnology, Santa Cruz, CA and Calbiochem, La Jolla, CA, USA) for fibronectin and tenascin, respectively. The bands were then visualized using enhanced chemiluminesence (NEN Life Science, Boston, MA, USA). The bands obtained were analyzed by densitometry.

The values obtained from the ECM (medium) had to be adjusted based on the total cell numbers at different treatment groups as the total protein estimation in the medium obtained from these different groups is dominated by the serum proteins in the complete medium and is not an accurate method of quantifying the secreted proteins which are in very small quantities. The total protein in the medium obtained from each treatment had approximately same value indicating that the serum proteins dominated the quantification. Thus, the same approach that was used for the quantitation of sGAG and HA (normalizing the values based on average total cell numbers utilized in the dose response experiment above) was followed. Briefly, the average cell numbers in the controls were taken as 100% and averages of cell numbers obtained

at different treatments were expressed as percentages of controls. These values were later used to normalize the densitometry values obtained from tenascin and fibronectin immunoblots in the ECM (medium). However, no such adjustment was needed for the blots obtained from whole cell lysates as equal and known quantities of the extract were loaded from each treatment.

Flow Cytometric Analysis of HEPM Cells Upon Exposure to SAD

Flow-cytometry

The HEPM cell cultures were established as described above. Upon attaining 20–25% confluence they were treated with IC_{50} of SAD daily for 48 h. The cells were harvested, washed with CMF-PBS containing 5 mM EDTA, re-suspended in 1 ml of PBS/EDTA and fixed in 100% ethanol for at least 30 min before staining. The cells were then washed with PBS followed by staining with a solution containing 5 mg/ml propidium iodide and 2 mg/ml of RNase in PBS. Following incubation for 30 min at 4°C, the cells were analyzed by flow-cytometry.

Statistical analysis

Data from at least three replicates for each parameter evaluated were analyzed for significance by one-way ANOVA and *post hoc* Student–Newman–Keuls (SNK) test. A p-value of ≤0.05 was considered significant.

Results

Reduction in HEPM cell numbers by SAD

In the control wells, HEPM cell number at the end of the experimental period was ~300 000/well. The cell number was significantly ($p \leq 0.05$) reduced by SAD in a dose dependent fashion (Fig. 1). Exponential regression analysis indicated that the 50% effective concentration (IC_{50}) for a reduction in cell number was 15.9 μg/ml of SAD. The minimal and no effect concentrations were 0.10 and 0.025 μg/ml of SAD, respectively.

Secalonic acid D inhibits the uptake of [^{3}H]thymidine by HEPM cells

Uptake of [^{3}H]thymidine (its presence in the acid-precipitable fraction of the cell homogenate), indicative of new DNA synthesis and cell proliferation, was significantly reduced by SAD in a dose dependent fashion (Fig. 2). The minimal and no-effect concentrations were 0.1 and 0.025 μg/ml of SAD, respectively.

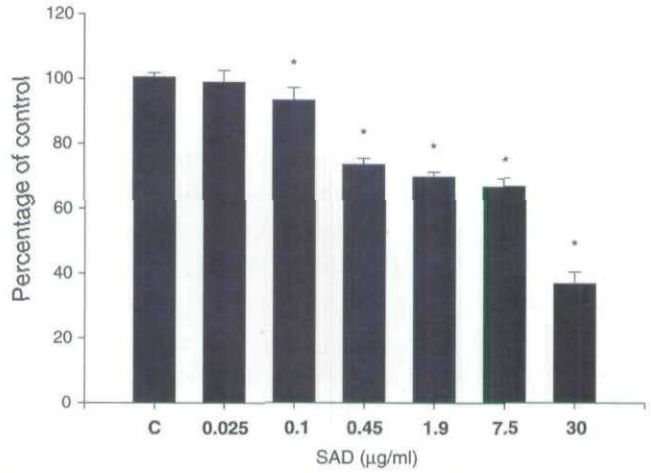


Fig. 1. Effect of secalonic acid D (SAD) on cell numbers. Cell numbers of human embryonic palatal mesenchymal (HEPM) exposed to SAD at concentrations of 0 (C), 0.45, 1.9, 7.5 and 30 μg/ml in the medium for 48 h. The numbers are expressed as percentage of control ± SEM (n = 4) on the *Y*-axis against the concentrations on the *X*-axis. Controls were taken as 100%; *Values significant at $p \leq 0.05$ when compared with control (0 μg/ml of SAD). A dose dependent decrease in the cell numbers could be observed compared with the controls. *Significant difference from the control.

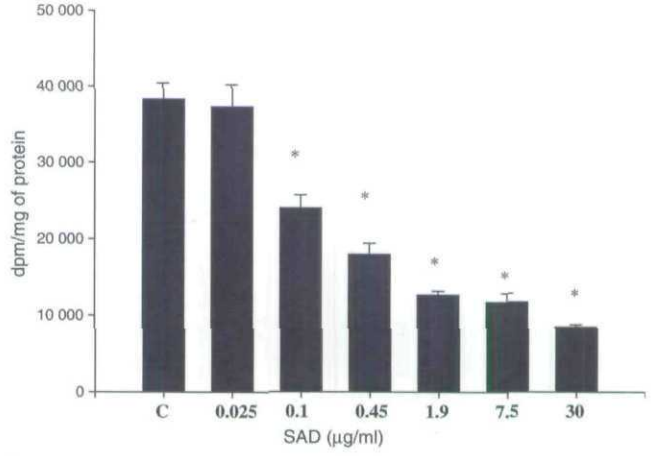


Fig. 2. New DNA synthesis taken as an estimate based on the amount of [^{3}H]thymidine uptake by cells when exposed to secalonic acid D (SAD). Human embryonic palatal mesenchymal cells were pulse labeled with [^{3}H]thymidine, 2 μCi/ml of culture medium for 4 h before the completion of the 48-h exposure period to SAD at concentrations of 0 (C), 0.45, 1.9, 7.5 and 30 μg/ml of medium (n = 3). The radioactivity counts are expressed as DPM/mg of protein ± SEM; *Values significant at $p \leq 0.05$ when compared with control (0 μg/ml of SAD). *Significant difference from the control.

Secalonic acid D promotes dye uptake (increases cell death) at higher concentrations

Uptake of trypan blue was noted in <7% of the cells in the control wells. The percentage of cells taking up the dye was significantly higher compared with the controls only at the two highest concentrations (7.5 and 30 μg/ml) of SAD tested (Fig. 3). The no-effect concentration was 1.9 μg/ml of SAD.

Secalonic acid D reduces the PCNA levels in the nucleus of HEPM cells

The nuclear PCNA level, considered very important for the synthesis of new DNA, is also widely used as a marker for proliferation. Western blot analysis of 5 μg of nuclear extracts obtained from HEPM cells treated with vehicle or IC_{50} of SAD (with respect to cell number) indicated a clear decrease in the levels of PCNA at the IC_{50} concentration (Fig. 4). Densitometric quantitation of the bands showed a reduction in nuclear PCNA protein level at this level of SAD exposure (Fig. 4). However, the level of PCNA in the whole cell extract was not affected by SAD (not shown).

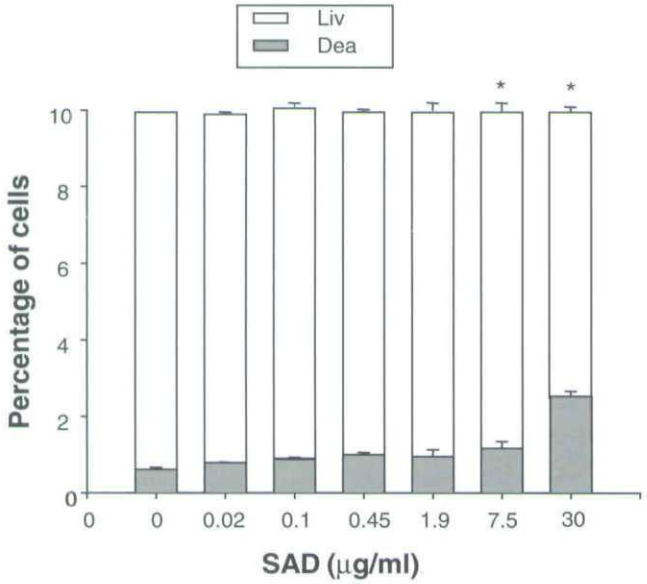


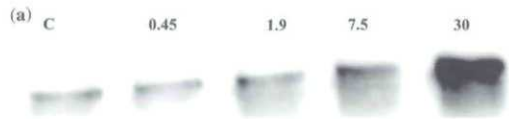
Fig. 3. Cell viability estimate based on trypan blue dye uptake (n = 3). Human embryonic palatal mesenchymal cells exposed to 0 (C), 0.45, 1.9, 7.5 and 30 μg/ml of secalonic acid D (SAD) in the medium for 48 h were harvested by trypsinization and stained with trypan blue. Cells taking up the dye (stained) were considered as dead and those not taking up the dye were considered viable. Numbers of live and dead cells were obtained by counting on the hemocytometre and expressed as percentages ±SEM taking their combination at each treatment as 100%; *Values significant at $p \leq 0.05$ when compared with control (0 μg/ml of SAD). Significant cell death was observed at 7.5 and 30 μg/ml of SAD compared with the controls. *Significant difference from the control in the percentage of both live and dead cells.

Control	SAD
27891 ± 1011	8097 ± 1251 *

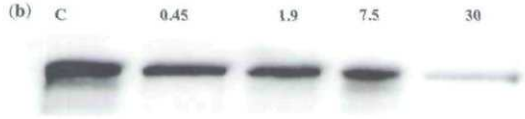
Fig. 4. Effect of secalonic acid D (SAD) on the levels of proliferating cell nuclear antigen (PCNA) in the nucleus 0.5 μg nuclear extracts obtained from human embryonic palatal mesenchymal cell cultures exposed to 0 (C) and 11.8 μg/ml of SAD (ED50) for 48 h were used for immunoblots for PCNA with anti-PCNA antibodies on 4–15% denaturing gel (n = 3). A clear reduction in the levels of PCNA was observed in the nucleus in the SAD treated samples compared with the controls. Band intensities are expressed as densitometric values ± SEM; *Values significant at $p \leq 0.05$ when compared with control (0 μg/ml of SAD). Effect of secalonic acid D on the level of PCNA in the nucleus. *Significant difference from the control.

Secalonic acid D does not inhibit ECM synthesis

Three major components of palatal ECM: fibronectin, tenascin and GAGs were quantified as a measure of ECM synthesis by HEPM cells. Western blot analysis showed no alteration in the quantity of fibronectin in the cell extracts following exposure to SAD except for a significant increase at the highest (30 μg/ml) concentration tested. Exposure to SAD resulted in a significant reduction in the level of fibronectin and tenascin in the extra cellular medium (Figs 5b and 6). However, for both proteins, this effect disappeared when the densitometry values were adjusted for the HEPM cell number at these dose levels (accompanying tables). No tenascin protein bands could be identified in the cell lysates containing up to 50 μg of protein. Exposure of [^{3}H]glucosamine-labeled HEPM cells to the highest

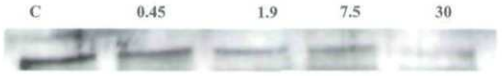


Control	0.45	1.9	7.5	30.0
11196 ± 1219	10376 ± 1464	10273 ± 1173	11878 ± 810	28944 ± 2109



Treatment	Control	0.45	1.9	7.5	30.0
Densitometric values	19870 ± 469	13272 ± 735	12512 ± 170	9622 ± 305	5310 ± 244
Adjusted densitometric values	19870 ± 469	18106 ± 1077	18183 ± 292	17141 ± 1856	16886 ± 2337

Fig. 5. Immunoblots (n = 3) for Fibronectin in the whole cell lysates (a) and extracellular matrix/medium (b). (a) 30 μg of whole cell lysate obtained from human embryonic palatal mesenchymal (HEPM) cell cultures treated with of 0 (C), 0.45, 1.9, 7.5 and 30 μg/ml of SAD in the medium, was used for immunobloting with anti-fibronectin antibody. No significant difference in the levels of fibronectin were noticed at any treatment level of SAD compared with the control except at the highest concentration. (b) 100 μg of protein in the extracellular matrix (medium) obtained HEPM cell cultures treated with of 0 (C), 0.45, 1.9, 7.5 and 30 μg/ml of SAD in the medium, was used for immunobloting with anti-fibronectin antibody. Although, a dose depended decrease in the levels of fibronectin was evident from the blots, the densitometry values that were normalized to the cell numbers (as shown in the methods) showed no significant reduction in the protein levels by SAD at any concentration. The normalized densitometry values ± SEM are shown below the blot. Effect of Secalonic acid D on the expression of Fibronectin.



Treatment	Control	0.45	1.9	7.5	30.0
Densitometric values	9933 ± 355	6698 ± 215	6040 ± 649	4208 ± 416	2617 ± 138
Adjusted Densitometric values	9933 ± 355	9090 ± 261	8770 ± 924	7515 ± 742	7765 ± 267

Fig. 6. Immunoblots (n = 3) for Tenascin in the extra cellular matrix (ECM)/medium. One hundred micrograms of protein in the ECM (medium) obtained human embryonic palatal mesenchymal (HEPM) cell cultures treated with of 0 (C), 0.45, 1.9, 7.5 and 30 μg/ml of secalonic acid D (SAD) in the medium was used for immunobloting with anti-tenascin antibody. A dose depended decrease, similar to that of fibronectin was also noticed in the levels of tenascin was noticed in the blots. A similar normalization (as mentioned in the methods) of the densitometry values indicated showed no significant reduction in the protein levels by SAD at any concentration. The normalized densitometry values ± SEM are shown below the blot. Effect of Secalonic Acid D on the expression of Tenascin Inhibition of G1 – S progression of HEPM cells by SAD. *Significant difference from the control.

concentration of SAD (30 μg/ml) did not result in a reduction in the levels of sGAGs and HA in the medium (Table 1).

Secalonic acid D inhibits the progression of cells from the G1 to S phase of the cell cycle

The culture conditions employed resulted in cycling of approximately 40% of the cells with ~20% each in 'S' and G2/M phases (Fig. 7). Exposure to SAD decreased the number of cycling cells (combined total number of cells in S and G2/M phases) by >30% with a concomitant increase in the number of cells in the G0/G1 phase. Greatest (42%) and significant ($p \leq 0.05$) reduction in the number of cells was seen in the 'S' phase with a tendency for reduction in their number in the G2/M phase.

Discussion

The mechanisms of pathogenesis of small palatal shelves and cleft palate by SAD (17) in mice were studied earlier using MEPM cells by Hanumegowda et al. (10). The results of their study showed that, although very high concentrations of SAD reduced ECM synthesis and cell viability, lower concentrations of SAD devoid of such effects inhibited the proliferation of the MEPM cells in culture. Their results suggested that SAD-induced reduction of palatal shelf size in mouse embryos is likely a result of a reduction in MEPM cell proliferation and number. The present study showed similar results in HEPM cells exposed to SAD with some quantitative differences. In the present study using HEPM cells, only 0.45 μg/ml SAD was required for a 25% reduction in cell number, compared to 1.9 μg/ml of SAD required for MEPM cells in the study by Hanumegowda et al. (10). Similarly, a 40–50% reduction in the *de novo* DNA synthesis (measured by the [^{3} H]thymidine uptake) required 0.45 μg/ml of SAD in HEPM cells in this study compared to 1.9 μg/ml in MEPM cells (10). These differences suggest that HEPM cells are approximately fourfold more sensitive to the anti-proliferative effects of SAD compared with MEPM cells, and seems to offer support to the downward adjustment of no effect levels for human exposure based on animal experiments using uncertainty/modifying/safety factors.

Table 1. **Effect of Secalonic acid D (SAD) on the synthesis of sulfated glycosaminoglycans and hyaluronic acid by the human embryonic palatal mesenchymal cells**

Hyaluronic acid (dpm/million cells)		sGAGs (dpm/million cells)	
Control	SAD (30 μg/ml)	Control	SAD (30 μg/ml)
33426 ± 1230	49611 ± 4636*	12992 ± 2052	11984 ± 2130

*Significant difference ($p \leq 0.05$) from the control for the same parameter.

Inhibition of G1 – S progression of HEPM cells by SAD.

Treatment	GO/G1	S	G2/M
Control	60.47 ± 1.5	20.49 ± 0.36	19.03 ± 1.4
SAD	73.04 ± 0.26*	11.91 ± 0.45*	15.09 ± 1.77

* indicates significant difference from the control

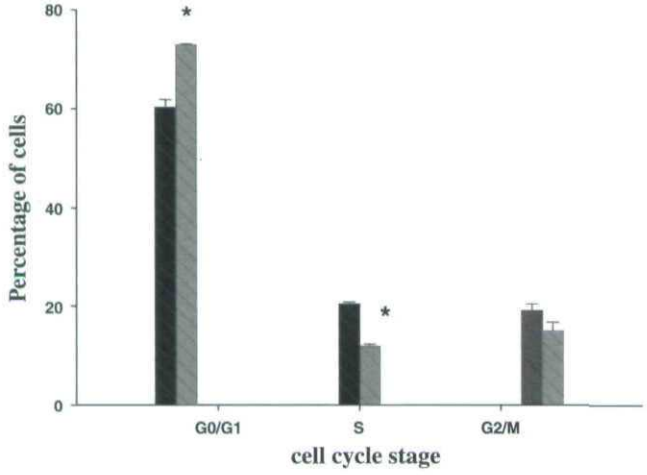


Fig. 7. Flow cytometric analysis (n = 4) of the effect of secalonic acid D (SAD) on the distribution of human embryonic palatal mesenchymal cells in the various cell cycle stages (G0/G1, S, G2/M) compared with the control. The percentages of cells in each cell cycle stage in the control and treated groups are shown in the table, along with SEM. The numbers are also represented in the graph where solid striped bars represent the controls while empty striped bars represent the SAD treated group. SAD caused a G1/S block as shown by a reduction in the number of S phase cells with a corresponding increase in the G0/G1 phase cells compared with the control group.

The conclusion that palatal mesenchymal cell proliferation is inhibited by SAD is supported by the significant reduction in the levels of PCNA in the nuclei of the SAD-treated HEPM cells compared with that in control cells. The nuclear migration of PCNA is essential for cell proliferation because of its role in DNA replication and repair during the late G1 and S phases of the cell cycle (18). Thus, the effect of SAD on the nuclear PCNA level in HEPM and MEPM cells in this and previous studies (10) respectively, suggests a likely effect for SAD on the events occurring in G1/S phases of the cell cycle. Mitogens such as epidermal growth factor and various signaling pathways such as the mitogen-activated protein kinase, PKC, PKA have been implicated in regulation of proliferation in palatal cells (14, 19, 20). As SAD is known to affect both PKC (11) and PKA pathways (12), delineation of the relative importance of each of these pathways is critical for the development of preventive measures against SAD-induced and other chemical-induced cleft palate.

As a significant level of cell death (as determined by trypan blue uptake) was induced by SAD in HEPM only at 7.5 μg/ml of SAD or higher (30 μg/ml of SAD) and as the minimal effective concentration for a reduction in cell number is well below this concentration (0.1 μg/ml), it can be assumed that the reduction in cell numbers at lower concentrations (below 7.5 μg/ml) is mainly a result of effect(s) other than SAD-induced cytotoxic effect.

The synthesis and localization of ECM components such as HA, fibronectin and collagen are important not only in maintaining the size and confirmation of the developing palate but also in its reorientation (21). These and other components such as tenascin may play a role in anchoring of the palatal mesenchymal cells to each other and in medial edge epithelial cell changes associated with palate fusion (21). Exposure to cleft palate-inducing agents such as the glucocorticoids and gene mutations (e.g. brachymorphic Br–/Br– mice) that impart susceptibility to cleft palate are associated with alterations in the synthesis of ECM components (22, 23). Hanumegowda et al. (10), however, failed to show any inhibitory effect of SAD on the synthesis of ECM in MEPM cells. In the present study, the quantities of sGAG and HA were unaffected by SAD both in the medium as well as in cell extracts. Although exposure to SAD resulted in a concentration-dependent decrease in fibronectin and tenascin in the medium, a pattern similar to that seen with HEPM cell numbers, the fact that the expression of these proteins in HEPM cell lysates was unaltered by SAD except at the highest concentration, suggests that SAD does not affect the synthesis of these ECM components on a per cell basis and that the observed reduction in fibronectin and tenascin in the medium is a result of reduced HEPM cell number. At 30 μg/ml of SAD, however, the possi-

bility that SAD may inhibit the transport of fibronectin from within the cells to the extracellular medium is suggested by an increase in intracellular levels of this protein.

In addition to the expected quantitative differences in components of cAMP signaling pathway, other investigators have shown diametrically opposed, i.e. stimulatory vs. inhibitory, proliferative responsiveness in HEPM compared with that in MEPM cells to exogenous TGF-β (14, 24). Surprisingly, however, results from our current study showed that SAD inhibits proliferation in both cell types in a similar fashion. If SAD inhibits TGF-β pathway, as is the case with the PKA and PKC pathways that regulate proliferation, such an effect should reflect as reduced proliferation in HEPM cells and as increased proliferation in MEPM cells. Whether such a lack of concordance between TGF-β responsiveness and the effect of SAD on proliferation among both cell types indicates a greater role for a common pathway other than that of the TGF-β in SAD-induced CP or different mechanistic targets in the two cell types is unknown. However, the fact, that both HEPM and MEPM exhibit similar responsiveness to SAD not only in cell proliferation but also in the synthesis of the ECM components studied, supports the former possibility.

To conclude, the results of these studies showed that the cleft palate-inducing agent, SAD, inhibits HEPM cell proliferation but not ECM synthesis at concentrations devoid of cytotoxic effects, suggesting that inhibition of palatal mesenchymal cell proliferation may contribute to its cleft palate induction. In addition, these studies showed that HEPM cells respond to SAD in a fashion qualitatively similar to, but quantitatively more sensitive than, that of MEPM cells in culture suggesting the relevance of our previous results with MEPM cells and thus mice, *in vivo*, to HEPM and thus likely to cleft palate in humans. Although differences between HEPM and MEPM cells in their responsiveness to growth factors such as TGF have been noted (14), the similarity of effects of CP-inducing toxicants in the two cell systems would, in addition to attributing human relevance to the animal teratogens shown to affect HEPM cell proliferation, allow further study of the functional significance of affected gene function following their stable overexpression or silencing in the HEPM cell line.

Acknowledgements: We would like to thank Dr Julia Taylor for her help with the experiments in this study. This study was funded by a grant from the Alternatives Research and Development Foundation.

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