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The effects of testosterone on craniosynostotic calvarial cells: a test of the gene/environmental model of craniofacial anomalies

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Structured Abstract

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Introduction – The gene–environmental interaction model for craniofacial development proposes that if a genetic predisposition for an anomaly is coupled with an environmental factor that can exacerbate this predisposition, more severe phenotypes will result. Here, we utilize cells derived from our non-syndromic rabbit model of craniosynostosis to test the hypothesis that an insult, testosterone (TP) administration (exogenous source) will alter the osteogenic activity of these cells.

Design – Calvarial cells from wild-type (WT) (N = 13) or craniosynostotic (CS) rabbits (N = 11) were stimulated with TP, an androgen receptor blocker, flutamide, and combined treatments. Proliferation and differentiation assays were conducted after 7 days. ANOVA and *t*-tests were used to determine differences in stimulation and cell type.

Results – The CS cells had significantly greater proliferation after TP administration compared to WT. There were no appreciable changes in differentiation after TP stimulation. Flutamide administration or combined TP and flutamide administration decreased both proliferation and differentiation for both cell types similarly.

Conclusions – Testosterone exposure caused an increase in cell proliferation for CS osteoblast cells. However, a therapy targeted to mitigate this response (flutamide therapy) similarly affected CS and WT cells, suggesting that the administration of flutamide or TP in the presence of flutamide decreases osteogenesis of these cells. Thus, although our data support a mechanism of gene–environmental interaction, these results would not support a therapeutic intervention based on this interaction.

Key words: craniosynostosis; gene/environment model; osteoblasts; sex steroids

Introduction

The gene–environmental model for the interaction in craniofacial development proposes that if a genetic predisposition for an anomaly is coupled with a environmental factor that can exacerbate this predisposition, more severe phenotypes will result (1). Cranial growth and development is largely genetically based (2, 3). In addition to genetic

factors, environmental influences contribute to craniofacial growth variations. These environmental influences include diet and dietary transition, temperature, muscular development, and biomechanical loadings (4, 5). However, the effect of environmental influences on the phenotypic spectrum of craniosynostosis is unknown.

Craniosynostosis is a pathological condition resulting from the premature fusion of the sutures of the skull. It involves the overgrowth of bone at the osteogenic fronts of the developing cranial bones. There is significant morbidity associated with craniosynostosis resulting from various secondary effects of suture fusion, including significantly elevated intracranial pressure (6), altered intracranial volume (6, 7), dilation of the subarachnoid spaces (7), optic nerve compression, papilledema, cognitive disabilities, and mental retardation (8–12).

Our group has studied a non-syndromic rabbit model of craniosynostosis. The rabbits of this colony demonstrate autosomal dominant transmission with a broad range of phenotypic expression (unilaterally affected animals, animals with delayed-onset suture synostosis, and animals with complete bilateral fusion) (13–16). Cells derived from the calvariae of this model are hypersensitive to osteogenic signals (17). Thus, this model is useful to study the environmental effects on pathology at the cellular level.

Craniofacial growth, development, and anomalies are influenced by hormonal regulation/dysregulation of the sex steroids (18–22), growth hormones (23–28), and thyroid hormones (29, 30). Environment influences endogenous hormone levels, including absorption via exposure from the environment. (31, 32). Recently androgen hormone regulation has been implicated to affect the growth, maintenance, and fusion of the calvarial sutures (18, 19, 33, 34). Additionally, dysregulation of androgenic hormone has been linked to craniosynostosis, both syndromic (Antley Bixler) and non-syndromic (anecdotal evidence suggesting a link to congenital adrenal hyperplasia and polycystic ovarian syndrome) (20, 35–39).

We test the hypothesis that testosterone (TP) administration alters the osteogenic activity of cells derived from the calvaria of a craniosynostotic (CS) animal model. We also test whether an androgen receptor blocker, flutamide, can block/ameliorate or mediate these effects. Given the paucity of work suggesting androgenic hormones modulate the osteoblast phenotype and suture fusion, we hypothesized that administration of an androgen receptor blocker would decrease the positive effects of TP on the CS rabbit cells.

Materials and methods

Calvarial bone samples were harvested from 10-day-old CS (n = 13) and wild-type (WT) New Zealand white rabbits (n = 11) according to a previously published protocol (17). Cells were isolated via two, 15-min digestions in 0.1% Collagenase Type I (Sigma Aldrich, St. Louis, MO, USA) in Hank's balanced salt solution. Explants of tissue and cells were grown in T-75 flasks in proliferation medium (Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin and 1% fetal bovine serum). At passage 2, cells were seeded in 96-well plates in triplicate at a density of 1000 cells per well. Absolute ethanol was used to reconstitute the TP, and serial dilutions were employed to create the concentrations used for experimental study.

To test the effects of androgenic hormone on control and experimental treatment, wells were set up for each plate and the treatments consisted of: 1) proliferation medium: control cells fed proliferation medium; and 2) TP reconstituted at concentrations of 1×10^{-12}, 10^{-14}, 10^{-16}, 1×10^{-20}, 10^{-24}, and 10^{-30} mM. This study kept each rabbit as an individual cell population. Treatments were run in triplicate wells (averaged), and the studies were run in triplicate.

To test the ability to modulate the effects of androgen exposure to these cells, flutamide (Sigma Aldrich), an anilide and androgen receptor blocker, was used as an experimental treatment for these studies. Absolute ethanol was used to reconstitute the flutamide, and serial dilutions were employed to create the concentrations used for experimental study. Control and experimental treatment wells were set up for each plate, and the treatments consisted of: 1) flutamide concentrations of 1×10^{-6}, 10^{-8}, and 10^{-10} mM; and 2) flutamide at above concentration with TP (1×10^{-16}). Again, this study kept each rabbit as an individual cell population. Treatments were run in triplicate wells (averaged), and the studies were run in triplicate.

Assessment of proliferation

Cell proliferation was determined by Cell-titer 96 Aqueous-One solution cell proliferation assay kit (Promega, Madison, WI, USA). After 7 days of treatment, cells were incubated for one hour with 20 μl per well of Cell-titer 96 Aqueous-One solution. The absorbance at 490 nm was recorded with a 96-well plate reader (Benchmark Plus; BioRad, Hercules, California, USA). Percent change in proliferation relative to baseline control measure was assessed for each treatment.

Assessment of alkaline phosphatase (ALP) activity

Cell differentiation was estimated using an ALP activity assay. ALP is an early biochemical marker for osteoblast differentiation. After 7 days of treatment, medium was removed from cells, and cell lysis was performed using Triton ×100 at 0.01% (Sigma). After 30 min of incubation at 4°C, deionized water and a p-Nitrophenyl phosphate solution were added to the lysis buffer. Three control wells containing no cells were also treated and served as blank controls to mathematically subtract the effects of the lysis buffer and water on final optical densities. Plates were incubated at room temperature in the dark for 30 min. The absorbance at 405 nm was recorded with a 96-well plate reader (Benchmark Plus; BioRad). ALP activity was then calculated using the following formula: ((optical density – the mean optical density of the control wells)*total volume*dilution)/(18.45*sample volume). Percent change in differentiation relative to baseline control measure was assessed for each treatment. All statistical analyses were performed using SPSS 17.0 (SPSS Inc, Chicago, IL, USA).

Results

Baseline measure

Differences in baseline proliferation by phenotype were assessed using a Student's *t*-test. After 7 days in culture, WT samples had greater mean proliferation (x = 0.8231, SE = 0.0594) than the CS-derived samples (x = 0.5605, SE = 0.0770, t = 2.700, p = 0.010). There was no significant difference in baseline ALP activity between WT and CS-derived samples. Increased cell number may cause the detection of increased ALP expression simply by assaying more cells. Therefore, percent change in ALP activity over control was used to compare the effects of hormone administration on these cells.

Testosterone administration

Change in proliferation after TP administration compared to baseline was determined using a paired *t*-test. There were no significant differences detected. Differences in percent change in proliferation by phenotype (WT vs. CS) and TP dose (−12 through −30) were assessed using a two-way ANOVA. A natural log transformation allowed for the assumptions of ANOVA to be met. The two-way interaction term was not significant. The main effect for dose was significant (F = 6.262, $p < 0.001$). Post hoc Bonferroni analysis revealed the −30 to have a greater percent change, increase, than all other doses ($p < 0.001$). The main effect of phenotype was significant (F = 9.663, p = 0.002). The CS-derived cells showed an increase in proliferation (x = 108.848, SE = 3.688) compared to WT (x = 98.575, SE = 1.230) after TP administration (Fig. 1).

Change in ALP activity after TP administration compared to baseline was determined using a paired *t*-test. There were no significant differences observed. Differences in percent change in ALP activity by phenotype (WT vs. CS) and TP dose (−12 through −30) were assessed using a two-way ANOVA. A natural log transformation allowed for the assumptions of ANOVA to be met. Two-way interaction term and main effects were not significant. CS and WT had similar ALP activity after TP administration (Fig. 1).

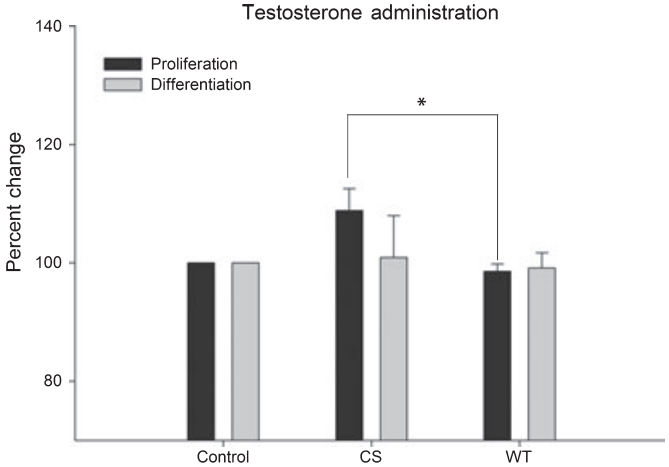

Fig. 1. Proliferation and differentiation after testosterone administration. Notice the increase in proliferation for craniosynostotic cells compared to the wild-type cells (*p = 0.002).

Flutamide administration

Change in proliferation after flutamide administration compared to baseline was determined using a paired *t*-test. Flutamide administration (x = 0.7533, SE = 0.0489) significantly decreased proliferation compared to baseline values (x = 0.8258, SE = 0.0518, t = 6.865, $p < 0.001$). Differences in percent change in proliferation by phenotype (WT vs. CS) and flutamide dose (−6 through −10) were assessed using a two-way ANOVA. All assumptions of ANOVA were met. Two-way interaction term and main effects were not significant. CS and WT had similar decreases in proliferation after flutamide administration (Fig. 2).

Change in ALP activity after flutamide administration compared to baseline was determined using a paired *t*-test. Flutamide administration (x = 0.0217, SE = 0.0018) significantly decreased ALP activity compared to baseline values (x = 0.0242, SE = 0.0019, t = 3.286, p = 0.002). Differences in percent change in ALP activity by phenotype (WT vs. CS) and flutamide dose (−6 through −10) were assessed using a two-way ANOVA. All assumptions of ANOVA were met. Two-way interaction term and main effects were not significant. CS and WT had similar decreases in differentiation after flutamide administration (Fig. 2).

Testosterone and flutamide administration

Change in proliferation after TP and flutamide administration compared to TP only was determined using a paired *t*-test. Flutamide added to TP administration (x = 0.7375, SE = 0.0392) significantly decreased proliferation compared to TP administration alone (x = 0.7921, SE = 0.0504, t = 3.016, p = 0.004). Differences in percent change in proliferation by phenotype (WT vs. CS) and flutamide dose (−6 through −10) were assessed using a two-way ANOVA. All assumptions of ANOVA were met. Two-way interaction term and main effects were not significant. CS and WT had similar decreases in proliferation after flutamide was added to TP administration (Fig. 3).

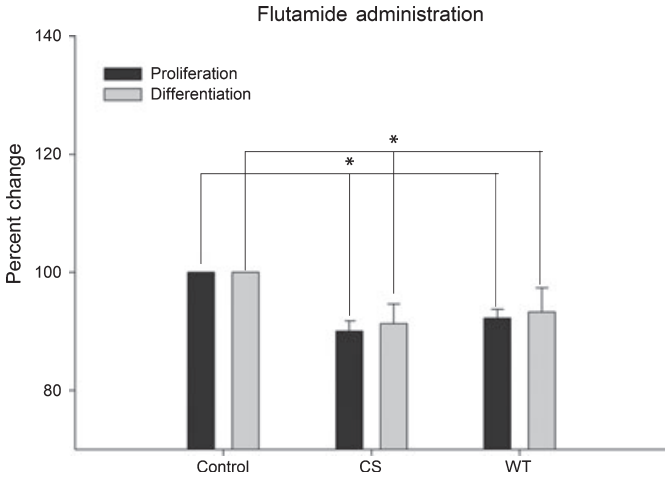

Fig. 2. Proliferation and differentiation after flutamide administration. Notice the overall decrease in proliferation and differentiation after flutamide administration (*$p < 0.001$ and p = 0.002, respectively).

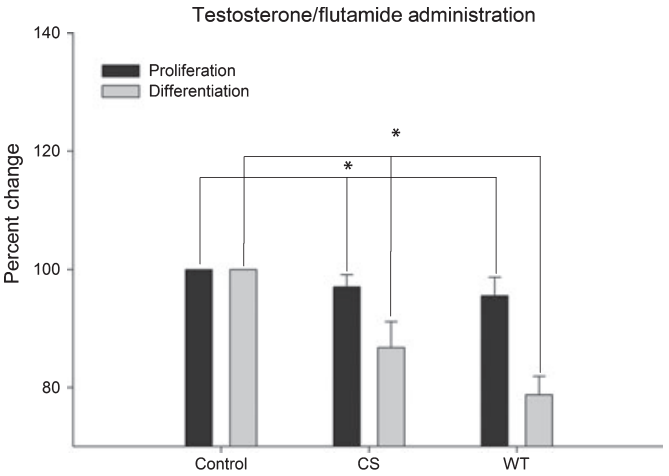

Fig. 3. Proliferation and differentiation after combined testosterone and flutamide administration. Notice the slight decrease in proliferation and the more marked decrease in differentiation after combined treatments (*p = 0.004 and $p < 0.001$, respectively).

Finally, change in ALP activity after TP and flutamide administration compared to TP only was determined using a paired *t*-test. Flutamide added to TP administration (x = 0.0195, SE = 0.0016) significantly decreased differentiation compared to TP administration only (x = 0.0244, SE = 0.0019, t = 5.693, $p < 0.001$). Differences in percent change in proliferation by phenotype (WT vs. CS) and flutamide dose (−6 through −10) were assessed using a two-way ANOVA. All assumptions of ANOVA were met. Two-way interaction term and main effects were not significant. CS and WT had similar decreases in ALP expression after flutamide was added to TP administration (Fig. 3).

Discussion

In this study, we hypothesized that TP administration would differentially affect the cells derived from the CS rabbit calvaria compared to cells derived from WT

controls. We further hypothesized that TP affected cells through the activation of androgen receptor, consistent with previous observations of the role of androgen receptors in mediating calvarial growth and suture fusion (18–20, 33, 34, 36). To test this hypothesis, flutamide, an androgen receptor blocker, was added to cells in culture.

There was a difference in the osteogenic response of calvarial-derived cells to TP by phenotype, with CS cells having a significantly increased proliferation following TP treatment. However, there was no phenotype difference observed for ALP activity, suggesting that TP: 1) exerted an effect on only one part of the cell cycle; 2) affected cell behavior at a critical time point in this study; or 3) proliferation and differentiation are controlled by different pathways. The differences observed in proliferation do support the gene–environmental model for craniofacial anomalies. Cells from a compromised phenotype, namely CS calvarial cells, have an exacerbated response to exposure to a mitogenic agent.

Interestingly, our data demonstrated a significant decrease in both proliferation and differentiation for both phenotypes after flutamide administration. These results suggest a role for the native androgen receptors during these portions of the cell life cycle within these cells. Results also suggest that blocking the androgen receptor can decrease the osteogenic response in calvarial-derived cells, corroborating data presented by Lin et al. (20, 36). However, differences between the CS- and WT-derived cells for these measures were not observed, suggesting that the decreased response was not specific to the compromised (i.e., CS) phenotype.

The combined administration of flutamide and TP also produced significant decreases in proliferation and ALP activity for both phenotypes. It is of interest to note that ALP activity was more greatly reduced, suggesting a greater effect for that portion of the osteogenic cycle. These data might suggest that when TP does not exert its paracrine effects through the androgen receptor, and therefore through another pathway (i.e., ERK, MAPK), it has an inhibitory effect for osteoblast differentiation. Again, strong phenotype differences were not observed, suggesting that the CS cells are not more susceptible to this combined treatment.

Differences observed between the different observed cell cycle markers, proliferation, and differentiation were differentially affected by the hormone challenges. These differences may be explained by: 1) differential effects of the hormones on the cell cycle or 2) an assay-based explanation where we may have increased proliferation of the bone-derived cells possibly including fibroblastic cells in our mixed population derived from the perisutural tissues. This is contrasted by the more specific results concerning the marker of ALP expression.

Testosterone exposure occurs both endogenously and exogenously (31, 32). Androgenic dysregulation is known to have disruptive effects on craniofacial growth and development (22, 35, 37–39). Lin et al. (36) demonstrated the presence of androgen receptors in dura mater and in cells of the osteogenic fronts and in the sutural mesenchyme of late gestation mice and human-derived suture tissues. These same authors also demonstrated that fetal calvarial osteoblasts and dural cells showed increased proliferation and differentiation after induced androgenic hormone expression, suggesting a possible role in suture fusion (20).

Craniosynostosis likely has a multifactorial threshold model, similar to that described for cleft lip and palate. The gene–environmental model of craniofacial growth and development suggests that if a genetic predisposition for an anomaly is coupled with an environmental factor, which can exacerbate the existing predisposition, a more severe phenotype will result. It has also been suggested that a better understanding of the gene–environment interaction could aid in the diagnosis and management of craniofacial anomalies (1).

Here, we suggest a possible mechanism for TP exposure causing increased cell proliferation in CS osteoblast cells. However, a therapy targeted to mitigate this response (flutamide therapy) similarly affected CS and WT cells, suggesting that administration of flutamide or TP in the presence of flutamide decreases osteogenesis of rabbit-derived calvarial cells in general. Thus, although our data support a mechanism of gene–environmental interaction, these results would not support a therapeutic intervention based on this interaction. This supposition is supported by an *in vivo* therapy targeting androgen receptor activity to delay suture fusion in this non-syndromic craniosynostosis rabbit model. Delivery of flutamide provided a transient rescue to suture fusion in this model (40). This further suggests that although exposure to hormones such as TP may be sufficient to cause an altered severe phenotype, these interactions may not provide useful information for therapeutic intervention. Rather

these gene–environmental interactions may only be sufficient to aid in identification and prevention of birth defects and growth anomalies.

Clinical relevance

Craniosynostosis is a pathological condition defined as the premature fusion of the sutures of the skull. The birth prevalence of the condition is estimated to be 300–500 cases per 1 000 000 live births. Cranial growth and development is largely genetically based. Environmental influences can also contribute to craniofacial growth variations. Here, we demonstrate that CS-derived bone-forming cells from our colony of naturally occurring non-syndromic CS rabbits increase cell number in the presence of excess or exogenous TP. In addition, an androgen receptor blocker decreased the osteogenic potential in these same cells. These results suggest that the dysregulation of androgenic hormone expression in the maternal environment may adversely affect those individuals susceptible to craniosynostosis. This principle may have important implications for those pregnant females suffering from hormone dysregulations, e.g., congenital adrenal hyperplasia or polycystic ovarian syndrome.

Financial disclosure and products page

There are no financial disclosures.

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