Quantitative analysis of Ah receptor and ARNT mRNA in cultured human embryonic craniofacial tissue.

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1. Introduction

The aryl hydrocarbon receptor (AhR) and the AhR nuclear translocation protein (ARNT) mediate the transcriptional regulatory responses of cells to polycyclic aromatic halogenated chemicals, of which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent. The AhR and ARNT are expressed in human tissues and occur at high levels in human placenta[1]. In the human embryo, AhR and ARNT are expressed in epithelial and mesenchymal cells of the developing secondary palate. Immunohistochemical doublestaining revealed that individual cells may express either AhR or ARNT, or both proteins simultaneously[2]. The present study examines the levels of mRNA for these proteins in cultured human craniofacial tissues. The levels of mRNA were quantitated by RT-PCR using a synthetic RNA internal standard specifically designed for each gene examined. The goal of the study was to quantitate the levels of AhR and ARNT in human embryos, to evaluate responses to TCDD (if any), to define any correlation between AhR and ARNT mRNA expression, and to examine the range of expression and/or response within this sample population.

2. Materials and Methods

Human embryonic craniofacial tissues were shipped overnight in CRCM-30 medium (American Type Culture Collection) at 4°C from the Human Embryology Laboratory, University of Washington, Seattle, WA. Tissues from 23 embryos ranging in gestational age from 42 to 54 days, a period encompassing development of the secondary palate, were examined. On arrival the tissues were examined by dissecting microscope, the left and right halves of the craniofacial region were separated and a sample of tissue from each anterior hard palate was collected. The remaining facial tissue was placed in culture, left side in control medium and right side in TCDD-containing medium (the assignment of side to treatment alternated so that left and right were equally represented in control and treated cultures). Tissues from 18 embryos were cultured in control and TCDD-containing medium (6 each at 1×10^{-10}, 1×10^{-9}, and 1×10^{-8} M TCDD) and samples were collected from each palate at 0, 2, 4, and 6 hours. Regions collected were anterior hard palate at 0 hours, mid hard palate at 2 hours, posterior hard palate at 4 hours, and soft palate at 6 hours. Craniofacial tissues from 2 embryos were cultured for 48 hours with collection of samples at 4, 10, 24, and 48 hours. Finally, craniofacial tissues from 3 embryos were cultured in control medium and all 8 regions (anterior, mid, and posterior hard palate, and soft palate; left and right sides) collected at 4 hours. Palatal organ culture was performed as described previously[3]. Total RNA

was prepared from each tissue sample by extraction with TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), followed by incubation of the aqueous layer of extracted reagent with RNA Tack Resin (Biotecx, Laboratories, Inc., Houston, TX) to bind total RNA. The resin-bound RNA was washed with ethanol, dried, and eluted from the resin with 20 μl of RNase-free water. Total RNA concentration was determined by OD_{260} of a 1:10 dilution in a microcuvette (7 μl volume) using the GeneQuant Spectrophotometer (Pharmacia-Biotech, Cambridge, England).

Quantitative RT-PCR was performed using standard protocols for reverse transcription of RNA (the reverse primer was used for the RT step) and amplification of the resulting DNA (reagents from Promega Corporation, Madison, WI). Primers were synthesized (Genosys Biotechnologies, Inc., Woodlands, TX) for ARNT to give a 238 nucleotide (nt) product and for AhR to give a 296 nt product in the coding region of each gene. The RNA internal standards (IS) were designed to incorporate the ARNT or AhR primers with a PUC19 plasmid sequence as a spacer to result in RT-PCR products of 275 and 343 bases, respectively. Five concentrations of IS were loaded across 5 tubes and then a constant amount of sample total RNA was added to each tube. Blocks of 18 samples (control and treated) were amplified in each run of 90 reactions. The RT-PCR products were separated on MetaPhor agarose (FMC BioProducts, Rockland, ME) gels. The ethidium bromide-stained bands were imaged with the Hamamatsu Photonics System, (Bridgewater, NJ) and quantitated using NIH Image. The ratio of the IS peak to that of the sample RNA was calculated and regressed against IS concentration to give the level of mRNA in the sample (expressed as molecules of AhR (or ARNT) mRNA per 100 ng of total RNA). Replicate RT-PCR experiments, including multiple gel analysis of some PCR products, were performed for a number of samples to confirm findings. All data were included in the statistical analysis with experiment included as a test parameter.

Statistical analyses were performed on log transformed gene product concentrations. A mixed effects linear model was used in estimating and testing the effects of treatment and time point[4,5]. The model for both AhR and ARNT included the fixed effects DOSE X TREATMENT X HOUR and PCRDATE nested within DOSE, and a random effect for SUBJECT. This allowed for a separate intercept for each combination of dose (10^{-10}, 10^{-9}, 10^{-8} M TCDD), treatment group (control or treated) and time (0,2,4,6 hours), with an adjustment for any effect of PCR run. It also took into account the variation due to subject. Maximum likelihood estimation was used. Age of the embryo and side of the palate were not significant predictors of AhR or ARNT in this model and so were removed from the analysis. To look at the effect of time on the gene levels, each treatment by time mean was tested for difference from the appropriate (control or treated within the dose level) zero hour mean. Treatment effect was tested by testing the difference between the treated and control groups within each time point and dose group. Differences between the treated and control groups in the change from the zero time point were also tested, to adjust for differences in the two sides of the palates. All comparisons were performed in two steps. First, pairwise t-tests were run within the mixed effects linear model described above. Since sample sizes were small and the asymptotic tests might not be correct, simulations were run to evaluate the results from the first step. For each gene, 200 data sets were created by drawing random samples with replacement from the original data set. The simulated data sets retained the structure of the original data set except that the sides of palates were randomly reassigned to treatment or control groups. The treatment comparisons were rerun in each of the data sets, and the probability of each original result was examined. Similarly, 200 data sets were simulated with hour being reassigned randomly, and the original results of the time comparisons were compared with the simulated results.

3. Results

The estimated means and confidence intervals from the linear model are shown in Table 1. After 2, 4 and 6 hours in culture, the AhR levels were significantly elevated compared to the 0 hour value for many of both the treated and control samples for all dose groups ($p<.05$ original data sets, $p<.1$ in the simulations). AhR at 6 hours was increased relative to 0 hour, but tended to be lower than the 4 hour level in most groups. For ARNT, only one group showed a time related effect, the control group in the Control/TCDD (1×10^{-8} M) set, was significantly lower at 4 hours compared to the 0 hour control value ($p=0.043$ original data, $p=0.030$ simulation).

There were no significant treatment effects with ARNT, and for AhR there were only a few significant treatment related contrasts. TCDD significantly reduced AhR expression at the 2 hour timepoint in the 1×10^{-9} M exposure group ($p=0.018$ original data, $p=0.05$ simulations). Also, TCDD exposure significantly increased AhR mRNA in the 1×10^{-8} M group, at 2 hours ($p=0.005$ original data, $p=0.085$ simulations) and 6 hours ($p=0.022$ original data, $p=0.07$ simulations).

In evaluating the potential for "high" or "low" expressors of AhR or ARNT, the ratios of treated to control samples were examined by individual as well as the estimates of the random effects of the individuals. Random effects estimates represent the proportionate change in the level of AhR or ARNT due to subject after adjusting for the effects of treatment, time, dose group and PCR run. Based on this analysis, the response to treatment is fairly consistent across subjects, and there is not a subset of individuals that can be considered high or low responders to treatment or expressors of either gene.

There is a partial correlation between AhR and ARNT, which after adjusting for any association due to concentration group, treatment or time, is very high: $rho=0.72$, $p<0.001$. Overall, the expression of AhR was approximately 3.9 times greater than that of ARNT.

Regional differences were not detected after culture in control medium for 4 hours. No significant differences between regions (anterior, mid, or posterior hard palate or soft palate) or sides (left or right) was detected. Also two embryos were cultured for a longer period with sampling at 4, 10, 24, and 48 hours. The outcome and conclusions from this data set were substantially the same as those from the larger data set and no meaningful treatment effects were found at the later timepoints.

4. Summary

In human developing palates, AhR increased with time from 0-6 hours, while levels of ARNT remained uniform. There was no effect of TCDD exposure on ARNT mRNA in these tissues. AhR may have slightly and significantly increased at 2 and 6 hours following exposure at 1×10^{-8} M TCDD. Expression of AhR and ARNT are partially correlated and overall the level of AhR was approximately 3.9 times that of ARNT. There were no regional differences in expression of AhR or ARNT in the human craniofacial tissues. Extending the culture period to 48 hours did not produce a significant treatment effect or substantially alter conclusions based on the 0-6 hour cultures.

Table 1: Estimates of Means* and 95% Confidence Intervals

Dose	10^{-10}		10^{-9}		10^{-8}	
	Mean	Conf. Interval	Mean	Conf. Interval	Mean	Conf. Interval
Gene = AHR						
C0	2.00	(1.13, 3.53)	1.05	(0.62, 1.79)	1.53	(0.91, 2.55)
C2	7.53	(4.41, 12.88)	3.11	(1.77, 5.45)	1.45	(0.82, 2.55)
C4	9.21	(4.83, 17.55)	4.31	(2.45, 7.61)	1.91	(1.08, 3.38)
C6	2.44	(1.41, 4.24)	3.62	(2.04, 6.44)	2.08	(1.19, 3.64)
T0	2.53	(1.40, 4.55)	0.85	(0.48, 1.51)	0.84	(0.50, 1.43)
T2	4.01	(2.28, 7.06)	1.52	(0.82, 2.80)	2.26	(1.27, 4.02)
T4	6.72	(3.66, 12.34)	2.92	(1.65, 5.17)	2.26	(1.24, 4.12)
T6	4.34	(2.47, 7.64)	3.16	(1.80, 5.53)	2.58	(1.54, 4.33)
Gene = ARNT						
C0	1.11	(0.64, 1.92)	0.83	(0.47, 1.47)	0.83	(0.47, 1.47)
C2	1.74	(0.97, 3.09)	1.17	(0.65, 2.09)	0.66	(0.36, 1.22)
C4	1.21	(0.65, 2.25)	0.82	(0.47, 1.42)	0.40	(0.21, 0.75)
C6	0.76	(0.44, 1.31)	0.69	(0.35, 1.36)	0.51	(0.28, 0.93)
T0	1.54	(0.83, 2.87)	0.41	(0.21, 0.77)	0.40	(0.21, 0.77)
T2	1.15	(0.66, 1.98)	0.58	(0.33, 1.01)	0.75	(0.42, 1.37)
T4	0.86	(0.50, 1.48)	0.38	(0.20, 0.70)	0.52	(0.27, 1.00)
T6	1.12	(0.70, 1.81)	0.46	(0.24, 0.88)	0.44	(0.24, 0.81)

* All values are in units: 10^6 molecules/100 ng RNA. C=control, T=TCDD-exposed, 0, 2, 4, 6= hours after start of culture. E.g.: C4=control collected at 4 hours.

5. References

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