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Patterns of some extracellular matrix gene expression are similar in cells from cleft lip-palate patients and in human palatal fibroblasts exposed to diazepam in culture

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In memory of emeritus Prof. Paolo Carinci, great scientist and expert embryologist.

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ABSTRACT

Prenatal exposure to diazepam, a prototype sedative drug that belongs to Benzodiazepines, can lead to orofacial clefting in human newborns. By using real-time PCR, in the present study we investigated whether diazepam elicits gene expression alterations in extracellular matrix (ECM) components, growth factors and gamma-aminobutyric acid receptor (GABRB3), implicated in the coordinate regulation of palate development. Palate fibroblasts were treated with diazepam (Dz-N fibroblasts) and compared to cleft lip-palate (CLP) fibroblasts obtained from patients with no known exposure to diazepam or other teratogens. Untreated fibroblasts from non-CLP patients were used as control. The results showed significant convergences in gene expression pattern of collagens, fibromodulin, vitronectin, tenascin C, integrins and metalloprotease MMP13 between Dz-N and CLP fibroblasts. Among the growth factors, constitutive Fibroblast Growth Factor 2 (FGF2) was greatly enhanced in Dz-N and CLP fibroblasts and associated with a higher reduction of FGF receptor. Transforming Growth Factor beta 3 (TGFβ_3) resulted up-regulated in CLP fibroblasts and decreased in Dz-N fibroblasts. We found phenotypic differences exhibited by Dz-N and CLP fibroblasts in GABRB3 gene regulation, so further studies are necessary to determine whether GABAergic system could be involved in the development of diazepam mediated CLP phenotype. Taken together the results elucidate the molecular mechanisms underlying possible toxicology effects induced by diazepam. Counselling of women on the safety of diazepam exposure is clinically important, also for the forensic consequences.

1. Introduction

Psychotropic drugs such diazepam are widely used for treatment of anxiety. Potential developmental toxicity of diazepam was assessed in several studies (Laegreid, 1990; Laegreid et al., 1990; Dolovich et al., 1998). Other studies however have refuted these data (Hartz et al., 1975; Czeizel, 1987; St Clair and Schirmer, 1992; Bergman et al., 1992), leading to a considerable controversy about the use of benzodiazepines in pregnancy. Defective shelf growth, failed elevation, defective fusion or post-fusion rupture of the palatal shelves, determined by genetic and/or environmental factors, may cause cleft palate (Zimmerman, 1984; Jurand and Martin, 1994; Carinci et al., 2007). The malformation is a common congenital disease (1/1000 newborns approximately) in Caucasian population with characteristics of a genetically complex trait (Scapoli et al., 1998, 2002).

It has been reported that diazepam elicits its teratogenic effects causing a delay in palatal shelf elevation (Katz, 1988) probably by perturbation of neurotransmitter mechanisms (Zimmerman, 1985). Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system and its receptor (GABRB3), are in fact involved in normal palatal development (Zimmerman et al., 1985; Homanics et al., 1997; Scapoli et al., 1998, 2002) and it is well known that benzodiazepines potentiate the GABA effects on GABRB3 (Mehta and Ticku, 1999). Diazepam mimics GABA binding GABRB3 to inhibit shelf reorientation and cause cleft palate (Wee and Zimmerman, 1983; Lavoie and Twyman, 1996; Farrant and Nusser, 2005; Cossart et al., 2005).

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Genetic analysis and tissue specific expression studies provide evidence that several growth factors are involved in cell palate differentiation such as Transforming Growth Factor alpha (TGFα) and the three isoforms of Transforming Growth Factor beta (TGFβ) (Lee et al., 1993; Pezzetti et al., 1998; Bodo et al., 1999a; Lidral et al., 1998). Fibroblast growth factor 2 receptor (FGFR2) gene is another candidate gene recently associated with cleft lip and palate (Osoegawa et al., 2008; Riley and Murray, 2007). FGF signalling previously known for its involvement in craniofacial development (Nie et al., 2006; Bodo et al., 1999a) is now in fact implicated in the genetic basis of both syndromic (associated to other abnormalities) and non-syndromic cleft palate (not associated to other diseases) (Pauws and Stanier, 2007).

In previous studies, we showed marked in vitro differences in the fibroblast phenotype of non-syndromic cleft palate subjects (CLP fibroblasts) in terms of extracellular matrix (ECM) component production (Bodo et al., 1999a; Bosi et al., 1998; Baroni et al., 2003, 2006). Based on these studies, we showed in an in vitro system diazepam effects on expression pattern for ECM components involved in the morphogenesis processes of palatogenesis, comparing them to CLP, to further define a putative involvement of diazepam in this common human malformation.

Using quantitative real-time PCR, gene expression of type I and VII collagens, vitronectin and tenascin C, biglycan and fibromodulin proteoglycans, integrins, disintegrin and MMP13 metalloprotease were analyzed in CLP fibroblasts obtained from patients with no known exposure to diazepam or other teratogens and in diazepam-treated l fibroblasts obtained from non-CLP patients (Dz-N fibroblasts) to identify convergent and/or divergent pathways. In order to clarify whether FGF2, TGFβ and their respective receptors could be involved in responses to their gene expression was also evaluated using the in vitro model. Finally, we examined gene encoding GABRB3 to hypothesise a possible involvement of GABAergic signalling in the teratogenic mechanisms of diazepam.

2. Materials and methods

2.1. Cell cultures

Fibroblasts were obtained from the oral flap edge of hard palate of 7-year old subjects with familiar non-syndromic cleft lip and palate during corrective surgery for the malformation (CLP fibroblasts). The pedigree collection of seven families has been extensively described in a previous report (Scapoli et al., 1997). All children were collected from regions of north eastern Italy and ascertained through the Archive for cleft lip and palate cases of the Clinic of Oral-Maxillofacial Surgery, General Hospital, Vicenza, Italy. All patients were non-syndromic and the use of clefting drugs in pregnancy was excluded. Informed consensus was obtained from all parents after having been informed about the nature of the study.

Control normal fibroblasts were obtained from the same anatomical site of 7 age-matched non-CLP subjects hospitalized for palate trauma. Human tissues were obtained with a protocol approved by our institutions. Informed consensus was obtained from all parents after the nature of the study had been fully explained.

Derived fibroblasts primary cultures from each subject (non-CLP and CLP), were separately grown in Iwaki flasks (Barloworld Scientific, Milan, Italy) containing Eagle's Minimum Essential Medium (MEM, Gibco, Paisley, UK) supplemented with 10% foetal bovine serum (FBS, Gibco, Paisley, UK), antimycotic and antibiotics (Gibco, Paisley, UK). Cultures were routinely monitored by phase contrast Leitz inverted microscope and fibroblast viability was measured by the cell's ability to exclude trypan blue. All tests were performed at the 8th subculture. For the assays, sub confluent control and CLP fibroblasts were incubated for 48 h in serum-free MEM alone. Parallel cultures of fibroblasts derived from each of seven non-CLP subjects, were treated with diazepam (N05BA01, Roche, in 96% ethanol) at the dose of 8 μg/ml (Dz-N fibroblasts). The dose, as reported in literature (Mino et al., 1994) may be correlated with the relative in vivo teratogenic potential of the drug. Untreated controls and CLP fibroblasts were maintained in medium containing 0.16% ethanol (final concentration).

2.2. 3H-thymidine incorporation

Control, Dz-N and CLP fibroblasts were collected and seeded at the density of 5×10^4 cells/ml in 1.9 cm^2 wells and maintained in MEM with 10% FBS, antimycotic and antibiotics until the subconfluence. After plating cultures were maintained for the following 48 h in serum-free MEM and 1 μCi/ml of ^{3}H-thymidine (Amersham International, Little Chalfont, England; s.a. 13.4 Ci/mmol) was added in the last 24 h. After incubation, the medium was discarded and the cells were tested according Marinucci et al., 2003. ^{3}H-thymidine results were expressed as cpm/10^6 cells; the cells were counted using the Burker's camera.

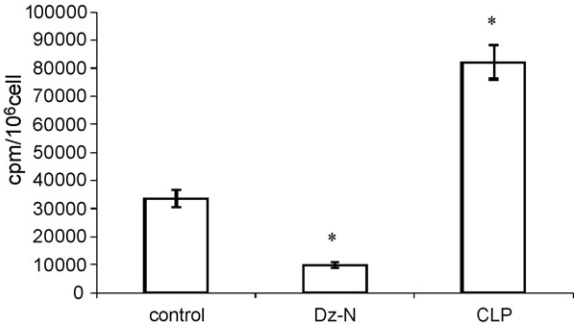

Fig. 1. ^{3}H-thymidine incorporation in control, Dz-N, CLP fibroblasts cultured for 24 h in MEM. Values were the mean ± S.D. of seven independent experiments, performed one for each of seven patients or normal subjects. Each experiment was performed in quintuplicate. Data were analyzed by analysis of variance (ANOVA). Differences vs. control: *F-test significant at 99%.

2.3. RNA isolation and real-time PCR analysis

Total RNA was isolated from control, Dz-N and CLP fibroblasts, by lysing the cells with Trizol reagent (Invitrogen, Paisley, Great Britain). RNA was quantified by reading the optical density at 260 nm. One microgram of total RNA was subjected to reverse transcription (RT) in a final volume of 20 μl using 200 units of M-MLV (Moloney murine leukemia virus) reverse transcriptase (Invitrogen Ltd., UK) for reaction with an oligo-dT primer (Invitrogen Ltd., UK). Real-time PCR was performed using 2 μl of the cDNA prepared by the RT reaction. The primer sequences of each gene are listed in Table 1.

Real-time PCR was carried out in a iCycler iQ PCR system (BioRad, Germany) using FAM for detection and ROX as a reference dye. One step PCR was performed in 25 μl of IQ SYBR Green Supermix (BioRad) according to the manufacturer's instructions. Product formation was monitored continuously with the fluorescent double-stranded DNA binding dye SYBR Green at each annealing step. The relative expression level of the housekeeping gene glyceraldehide-3-phosphate dehydrogenase (GAPDH) was used to normalize the marker gene expression in each sample in different concentrations. Immediately following the PCR, a melting curve was undertaken by raising the incubation temperature from 55 to 95 °C to confirm amplification specificity. All samples and the templates for the standard curves were run in triplicate.

2.4. Statistical analysis

Results reported in figures are the mean ± S.D. (standard deviation) of seven independent experiments, performed one for each of the seven patients or normal subjects. Real-time PCR was performed in triplicate for each subject, ^{3}H-thymidine incorporation in quintuplicate. Statistical analysis was performed by analysis of variance (ANOVA) followed by the Sheffe F-test.

3. Results

3.1. Cell morphology

Cultured fibroblasts from normal and cleft palate exhibited a continuous monolayer of viable fusiform-shaped cells. No differences in morphology and viability were observed after diazepam treatment (data not shown).

3.2. Cell proliferation

^{3}H-thymidine incorporation was significantly decreased in Dz-N fibroblasts (−70%) and markedly increased in CLP fibroblasts (+151%) when compared to controls (Fig. 1).