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Immunoexpression of GADD45β in the myocardium of newborns experiencing perinatal hypoxia

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ABSTRACT

Aim: Among the several organs affected by perinatal hypoxia, the heart plays a central role, with cell death caused mainly by apoptosis. One of the biomarkers most often linked to hypoxia-derived apoptosis of cardiomyocytes in animals is Gadd45β. From the published literature, Gadd45β is proposed as a biomarker of hypoxia-induced lesion in cardiomyocytes, both *in vitro*, as well as in animal models. Our study suggests that this protein can be used as an early biomarker of cell damage in neonate's cardiomyocytes (humans specimens), a process that can ultimately lead to apoptosis. The aim is to determine levels of tissue immunoexpression of the Gadd45β biomarker in myocardium samples of newborns affected by hypoxia, and to correlate these results with clinical and anatomopathologic data.
Methods: Myocardium samples from the left ventricle of newborns were used. The samples were collected from 78 autopsies performed in neonates of both genders, with hypoxia (Apgar score at five minutes below 6 and/or pH below 7.2 and/or autopsy with anatomopathological signs of hypoxia), who had died within the first day of life. All samples were organized in Tissue Microarray. Immunohistochemistry analysis, using anti-Gadd45β as the primary antibody, was performed on 3 multi-sample histological slides. There was no correlation between Gadd45β tissue immunoexpression and neonatal weight ($p = 0.93$), gestational age ($p = 0.16$), Apgar score at first minute ($p = 0.914$), Apgar score at five minutes ($p = 0.988$) and arterial blood pH ($p = 0.542$). There was a relation between Gadd45β tissue immunoexpression and survival ($p = 0.02$). The maximum peak of Gadd45β tissue immunoexpression was 8.43% HPF (high power field) and was observed around of six hours of life.
Conclusion: Gadd45β could be a suitable biomarker of cardiomyocytes apoptosis in newborns experiencing hypoxia in the first day of life, as its highest tissue immunoexpression around at the first six hours after birth.

1. Introduction

Hypoxia can be defined as inadequate cellular oxygenation, often associated with cyanosis, which can lead to cardiomyocyte death when affecting the heart [1]. This process of cell death, known as apoptosis, as well as the strategies behind cardiomyocyte recovery from perinatal hypoxia in newborn infants, is poorly understood.

A study performed in animals showed a clear link between perinatal adverse effects and an increased risk of myocardial ischemia in adulthood [3].

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The study of gene expression induced by stimuli such as hypoxia has highlighted the molecular signatures of these responses. One of the genes most recently found to associate with hypoxia-induced apoptosis is *Gadd45β*, a member of the *Gadd45* family [2]. Our results suggest that Gadd45β could be used as an early biomarker of hypoxia-induced apoptosis in the myocardium of newborn infants who had experienced perinatal hypoxia.

The aim of this study was to determine the tissue immunoexpression of the Gadd45β protein in samples from the myocardium (left ventricle) of newborns who had experienced hypoxic conditions and died within the first day of life; moreover, the study aimed to correlate these results with clinical and anatomopathological data.

2. Methods

2.1. Sample selection

Fragments of formaline fixed – paraffin embedded (FF-PE) myocardium (left ventricle) were obtained from neonates' autopsies (Division of Pediatric Autopsies of the Anatomic Pathology Service – Clinical Hospital, Federal University of Paraná) from 1991 to 2007. The number of the autopsy prior to sample access anonymized the patient information.

All the neonates who had developed hypoxia were included in the study (gestational age of 24 weeks or more), both preterm and full term, both gender, and who had died within the first 24 h of life. The clinical and anatomopathological criteria for hypoxia were defined as follows: (a) Apgar score below 6 at five minutes, and/or (b) arterial blood pH below 7.2, and/or (c) anatomopathological signs of acute perinatal hypoxia detected in the autopsy (petechiae in serous membranes and mucosae, multi-organ systemic congestion, alveolar hyperinflation, atelectasis, hypoxic-ischemic encephalopathy, among other systems). The exclusion criteria consisted of damaged samples and incomplete medical records of the newborn.

2.2. Sample classification

The following data were retrieved from the medical records: gender, weight in grams, gestational age in weeks, Apgar score at the first minute and at the fifth minute, arterial blood pH, and survival in hours of life. The anatomopathological records revealed the cause of death and the underlying disease. The cases were classified according to the variables described above.

2.3. Slide preparation

TMA (tissue microarrays) were mounted from the original paraffin blocks containing myocardium samples (left ventricle). Seven TMA, with an average of 5–6 cases, represented by 2 cores samples each, were prepared. Each core was represented by one circle myocardium sample with 3 mm in diameter. The total myocardium area analyzed was 14 mm2 ($A = \pi r^2 \times 2$ cores samples). The TMA blocks were sectioned to originate multi-sample slides that were analyzed by using immunohistochemistry.

2.4. Immunohistochemistry

The immunoperoxidase procedure was used in for immunohistochemistry, as described by Debur et al. [4]. Each immunostaining reaction included positive controls (myocardium samples of left ventricles from adults with ischemic disease) and negative controls (without incubation with the primary antibody). The primary antibody used was the rabbit polyclonal anti-Gadd45β from SIGMA® (St Louis, USA), at 1:100 dilution.

2.5. Immunohistochemistry analysis

The immunostained slides were observed using an optical microscope Olympus® BX50 (Tokyo, Japan), coupled to a Dino-eye video camera enhanced by image analysis software Image Pro Plus™ (Maryland, USA). For each sample, eight photomicrographs were taken in HPF (high power field = 400×), with a total area of 115,226.1 μm2 and with 1024 × 768 pixels each.

The positive control HPF photomicrography was chosen as the "mask", which contained adequate levels of positive tissue immunoexpression signal. Then, the mask was superimposed to the samples photomicrographs.

Based on the ideal positive tissue immunoexpression signal obtained from the mask, the image analysis software Image Pro Plus (TM) identified the positive areas in the samples and is able to transform these results into positive tissue immunoexpression area per square micron (μm2). The area in μm2 obtained with this method was divided by the constant 115,226.1 μm2, which is the total area of the HPF observed, thus generating a percentage value for the positive tissue immunoexpression area for each HPF. For each case, an average percentage of positive area was determined in eight HPF images.

2.6. Statistical analysis

To test the association between two qualitative variables, the exact Fisher test or the chi-square test was used. Comparison between two groups, concerning quantitative variables, was performed with the use of the Student's *t*-test for independent samples or the Mann-Whitney nonparametric test. Three groups were compared with the use of the one-way analysis of variance (ANOVA) model or the Kruskal-Wallis nonparametric test. For these comparisons, the analysis of covariance (ANCOVA) model was also used, where the weight at birth was considered a covariate. Values of $p < 0.05$ reflected statistical significance. The data were analyzed with the IBM SPSS Statistics v.20 software.

2.7. Approval by the research ethics committee

The Research Ethics Committee of the Federal University of Paraná under opinion number 2533.140/2011-06 approved this study.

3. Results

There was a slight predominance of males in the samples analyzed: 60% of the total (47 cases). Most of the cases were premature newborns (PNB): approximately 87% of the total (68 cases). The main cause of death was perinatal hypoxia, corresponding to 98% of the total (77 cases), whereas the main underlying disease was hyaline membrane disease, corresponding to 46% of the total (36 cases). The remaining results can be found in Table 1.

Gadd45β expression was detected in all samples (Fig. 1), with tissue immunoexpression ranging from 0.35% to 8.43% per HPF, with median value of 1.66% (Table 1).

Tissue immunoexpression of Gadd45β was correlated with the variables weight of newborn, gestational age, Apgar score at first minute of life, Apgar score at five minutes of life, and arterial blood pH; there were no statistical significances (Table 2).

Gadd45β tissue immunoexpression was also correlated with survival, in hours within the first day of life, with a highest tis-