Differential expression of Bcl-2 proto-oncogene in the trophoblast from embryos with Down's syndrome and those after spontaneous abortion

D. Tamiolakis[1], N. Papadopoulos[2], J. Manavis[3], G. Alexiadis[3], D. Karamanidis[4], A. Kotini[5],A. Bounovas[6], K. Simopoulos[6]

[1]Department of Cytology, Regional Hospital of Alexandroupolis; [2]Department of Histology-Embryology, Democritus University of Thrace; [3]Department of Radiology, Democritus University of Thrace; [4]Department of Obstetrics & Gynecology, Regional Hospital of Alexandroupolis; [5]Department of Medical Physics, Democritus University of Thrace; [6]Department of Experimental Surgery, Democritus University of Thrace (Greece)

Summary

Down's syndrome (trisomy 21) was the first human chromosomal syndrome to be recognized (in 1959 by Lejeune and colleagues). It is also the most frequent chromosomal aberration occurring in one out of 700 live newborns. In the present study we investigated the immunohistochemical expression of the apoptosis-suppressing protein Bcl-2 in placental trophoblastic cells from embryos with Down's syndrome (gestational age 12th, 15th and 22nd week) and correlated the findings with equivalent trophoblastic cells from embryos after spontaneous abortion. In our cases with Down's syndrome a weak Bcl-2 expression was noted in the cytotrophoblast and syncytiotrophoblast of chorionic villi in contrast to strong Bcl-2 staining of the same cells in the cases of spontaneous abortions ($p < 0.0001$). Although there are no specific findings that truly characterize a placenta with trisomy, obtaining a small piece of chorionic villus tissue (chorionic villus biopsy) and immunohistochemical control for Bcl-2 protein could be an additional prenatal examination available to the perinatologist to detect chromosomal abnormalities.

Key words: Trophoblast; Bcl-2; Down's syndrome; Spontaneous abortion.

Introduction

The trophoblast is the ectodermal covering of the blastocyst that erodes the uterine mucosa and through which the embryo receives nourishment from the mother. During placental development, various subsets of the trophoblast originate from the trophoectoderm of the blastocyst, including both the villous trophoblast, comprising the cytotrophoblast and syncytiotrophoblast covering the placental villi, and the extravillous cytotrophoblast of cell islands and cell columns. Cell islands and cell columns are composed of a stratified core of extravillous cytotrophoblast surrounded by a sleeve of syncytialtrophoblast [1]. Cell islands are free-ending structures in the intervillous space, whereas cell columns are responsible for the attachment of placental villi to the basal plate, forming the so-called anchoring villi. From the most distal parts of these villi, extravillous trophoblastic cells invade the decidua [2, 3]. Trophoblast is a potent source of steroid hormones (estrogen and progesterone) and protein hormones (human chorionic gonadotropin-hCG, and placental lactogen-hPL); it also plays an important role in facilitating immunologic tolerance of the fetal allograft (expression of class I antigen and interleukin 6-IL by intermediate trophoblastic cells and syncytiotrophoblastic cells, respectively).

An integral membrane protein that functions to suppress programmed cell death [4], 25-KD Bcl-2 is widely expressed during embryogenesis [5]. In an adult, however, endogenous Bcl-2 protein is normally restricted to long-living cells such as mature B and T lymphocytes, neurons, early hematopoietic progenitors, stem cell zones of intestine, epidermis, and hormonally regulated epithelium [6].

A reason for the accumulation of Bcl-2 positive cells may be, in part at least, the involvement of this protein in apoptosis, meaning PCD (programmed cell death), in some cells without affecting proliferation. The disregulation in the PCD process may trigger the malignant transformation of lymphoid cells.

Approximately 95% of all cases with Down's Syndrome (DS) harbor primary trisomy 21, about 4% harbor a translocation, and 1% a mosaic. The most frequent translocation is between chromosome 21 and a chromosome of the D (usually 14) or G (usually 22) groups. More than 50% of the D/G and 90% of the G/C translocations occur de novo, with both parents having normal karyotypes. The remaining cases with D/G or G/C translocation have a parent with the same translocation in a balanced form; a balanced translocation can be found in several members of such families. The risk of inheriting D/G translocation in the mother is 11% and in the father 2.4%. In the very instance of (21q; 21q) translocation the risk is 100% [7]. Infants with DS have intrauterine growth delay (IUGD). The phenotype of Down's syndrome can be recognized easily in the fetus as early as 14-weeks' gestation.

Several approaches are now available to the perinatologist for assessing the growth and development of the

Revised manuscript accepted for publication May 26, 2001

Fig. 1

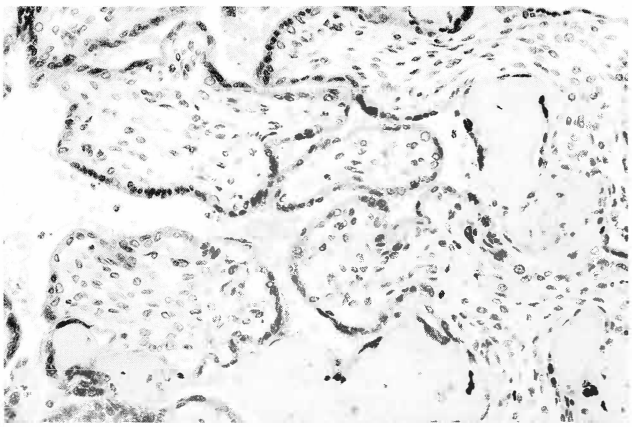

Fig. 2

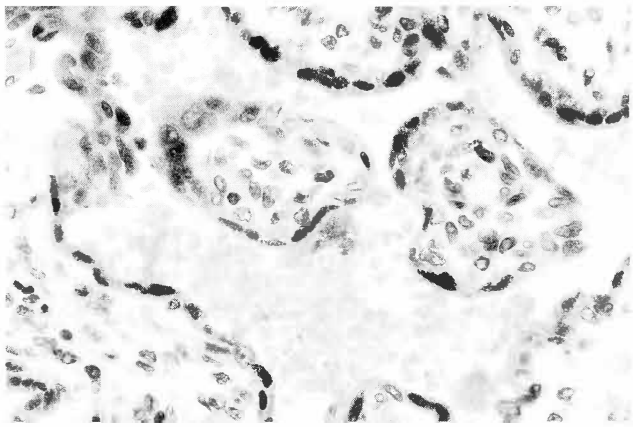

Fig. 3

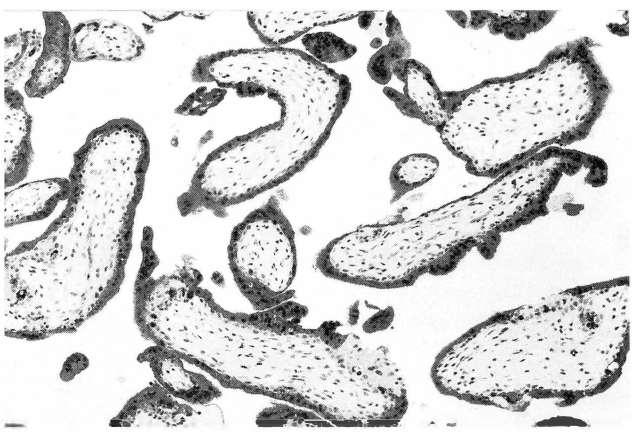

Fig. 4

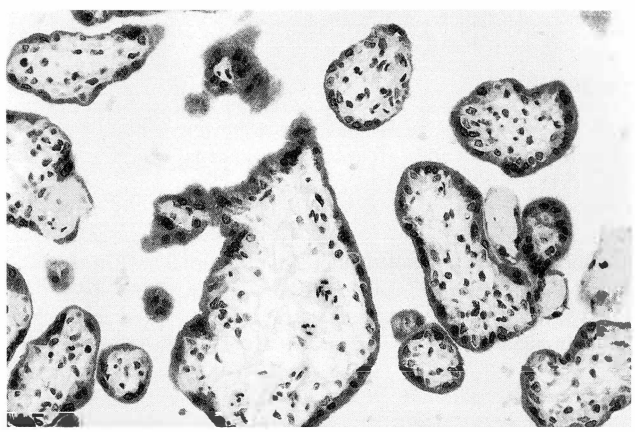

Figure 1. — Weak Bcl-2 staining is noted in the trophoblast (cytotrophoblast and syncytiotrophoblast) of chorionic villi (anti-bcl-2 x 100). Placenta at 12 weeks of gestation. Down's syndrome.
Figure 2. — Weak Bcl-2 staining is noted in the syncytiotrophoblast of chorionic villi (anti-Bcl-2 x 250). Placenta at 22 weeks of gestation. Down's syndrome.
Figure 3. — Intense Bcl-2 staining is noted in the the trophoblast (cytotrophoblast and syncytiotrophoblast) of chorionic villi (anti-Bcl-2 x 100). Placenta at 12 weeks of gestation. Spontaneous abortion.
Figure 4. — Intense Bcl-2 staining is noted in the syncytiotrophoblast of chorionic villi (anti-Bcl-2 x 250). Placenta at 22 weeks of gestation. Spontaneous abortion.

fetus in utero. In combination, these techniques are designed to detect malformations, chromosomal abnormalities, and overall growth of the fetus. The least traumatic of these procedures is ultrasonography [8] which employs ultrasound to produce images of the placenta and fetuses. Ultrasonograms can determine placental and fetal size and position, multiple births, and malformations such as neural tube defects. Another approach involves aspiration of amniotic fluid and is termed amniocentesis [9]. The fluid itself is analyzed for a-fetoprotein (AFP). Furthermore, fetal cells which are present in amniotic fluid grow in culture and can be checked for chromosomal abnormalities. In this manner major chromosomal alterations such as trisomies and monosomies can be identified.

A more recent technique involves obtaining a small piece of chorionic villus tissue (chorionic villus biopsy) [10]. This tissue contains numerous rapidly dividing fetal cells which are available for immediate analysis for chromosomal and biochemical defects.

In order to gain insight into the function of Bcl-2, we studied the distribution of its expression in the placental trophoblast of the chorionic villi (cytotrophoblast and syncytiotrophoblast) in embryos with Down's syndrome and those after spontaneous abortion at approximately 12, 15, and 22 weeks of gestation. The localization of Bcl-2 oncogene within the trophoblastic cells was determined using immunohistochemical methodologies and an anti-Bcl-2 monoclonal antibody.

To our knowledge, no data are available concerning Bcl-2 expression in the placental trophoblast in embryos with Down's syndrome in different stages of development and embryos after spontaneous abortion.

Materials and Methods

Samples representing 15 placentas from embryos with Down's syndrome and 15 placentas from embryos after spontaneous abortion were obtained at the 12th, 15th and 22nd week of gestation. Placentas were cut as thick as 3 mm, then fixed in 10% neutral buffered formaldehyde at 4°C for 24 hours and processed for routine paraffin embedding. Paraffin blocks were available in all cases and tissue sections were stained with hematoxylin-eosin (H-E), PAS and Giemsa.

Immunohistochemical stain for Bcl-2 (mouse monoclonal, clone 124, 1:20, Dako, GmbH Hamburg, Germany) was perfor-

med on 4-μm-thick sections of formalin-fixed and paraffin-embedded tissues with antigen retrieval for 30 minutes by microwave in a 10 mmol/l citrate buffer (PH 60) within a pressure cooker. After antigen retrieval, endogenous peroxidase was quenched by incubation of the sections with a solution of 3% H_2O_2 in methanol. The sections were then incubated overnight at room temperature with the primary antibody followed by a peroxidase labeled detection polymer (Dako Envision System, Dako) for Bcl-2 according to the manufacturer's instructions. Diaminobenzidine was used with hydrogen peroxidase as a chromogen. Sections were subsequently counterstained with hematoxylin and cover-slipped.

The percentage of Bcl-2 positive trophoblastic cells (cytotrophoblastic and syncytiotrophoblastic) was graded as follows: 0= none, 1=1 to 25%, 2=26 to 50%, 3=51 to 75%, 4=76 to 100%. Immunostaining intensity was rated as follows: 0=none, 1= weak, 2= moderate, and 3= intense.

Specimens were considered immunopositive when ≥1% of the trophoblastic cells had clear evidence of immunostaining. Finally, an immunoreactive score was calculated by multiplying the percentage of positive cells by staining intensity score, as proposed by Krajewska *et al.* [11]. In the case of heterogeneous staining intensities within one sample, each component was scored independently and the results were summed up. For example, when a specimen contained 50% of the trophoblastic cells with moderate intensity (2×2=4), 25% of trophoblastic cells with intense immunostaining (1×3=3), and 25% of cells with weak intensity (1×1=1), the score was 4+3+1=8.

Results

The sections were examined independently by two observers and scored according to the intensity of staining and proportion of stained cells. Positive staining for Bcl-2 protein was manifested as fine red cytoplasmic granularity (Figures 1-4). The immunohistochemical results are shown in Tables 1 and 2. Table 1 summarizes the results of the immunohistochemical analysis of Bcl-2 in the 15 cases of placentas from embryos with Down's syndrome (12th, 15th, and 22nd week of gestation) and Table 2 the 15 cases of placentas from embryos after spontaneous abortion. The immunostaining results were evaluated in terms of percentages of immunopositive placental cells (cytotrophoblastic, syncytiotrophoblastic cells) of the chorionic villi and relative immunointensity.

Table 1. — *Expression of Bcl-2 protein in trophoblastic cells from embryos with Down's Syndrome.*

Condition	N. of cases	Immunoreactive scores (average)	
		Cytotrophoblast	Syncytiotrophoblast
Fetal specimens			
12th week of gestation	5	5	5
15th week of gestation	5	6.5	6.5
22nd week of gestation	5		6.5

Table 2. — *Expression of Bcl-2 protein in trophoblastic cells from embryos after spontaneous abortion.*

Condition	No. of cases	Immunoreactive scores (average)	
		Cytotrophoblast	Syncytiotrophoblast
Fetal specimens			
12th week of gestation	5	10.5	10.5
15th week of gestation	5	10	10
22nd week of gestation	5		10

Down's syndrome

12th week of gestation

The cytoplasmic expression of Bcl-2 in placental cells (cytotrophoblastic, syncytiotrophoblastic) in our series showed two cases with weak (1+), one with moderate (2+) and two with strong (3+) staining patterns. Regarding the percentage of positive trophoblastic cells, two were listed in category 1 (1 - 25%), one in category 2 (26 - 50%) and two in category 3 (51 - 75%). The immunoreactive scores ranged between 1 and 9 (median 5) (Figure 1).

15th week of gestation

During this period Bcl-2 was expressed weakly in the majority of cytotrophoblastic cells, whereas in the syncytiotrophoblastic cells three cases with moderate (2+), and two with intense (3+) staining patterns were noted. With regard to the percentage of positive trophoblastic cells, three were listed in category 2 (26 - 50%) and two in category 3 (51 - 75%). The immunoreactive scores ranged between 4 and 9 (median 6.5).

22nd week of gestation

In the syncytiotrophoblastic cells two cases with moderate (2+) and three with intense (3+) staining pattern were noted. Regarding the percentage of positive cells, two were listed in category 2 (26 - 50%) and three in category 3 (51 - 75%). The immunoreactive scores ranged between 4 and 9 (median 6.5) (Figure 2).

Spontaneous abortion

Table 2 summarizes the results of the immunohistochemical analysis of Bcl-2 in the 15 cases of placentas from embryos (12th, 15th, and 22nd week of gestation) after spontaneous abortion. Trophoblastic cells demonstrated higher values of Bcl-2 expression correlated with those found in the cases of Down's syndrome.

12th week of gestation

The cytoplasmic expression of Bcl-2 in all our settings showed a strong (3+) staining pattern. Regarding the percentage of positive trophoblastic cells, two were listed in category 3 (51 - 75%), and three in category 4 (76 - 100%). The immunoreactive scores ranged between 9 and 12 (median 10.5) (Figure 3).

15th week of gestation

During this period cytoplasmic expression of Bbcl-2 showed in two cases moderate (2+), and three intense (3+) staining patterns. With regard to the percentage of positive cells, four were listed in category 4 (76 - 100%) and one in category 3 (51 - 75%). The immunoreactive scores ranged between 8 and 12 (median 10).

22nd week of gestation

The reactivity of the syncytiotrophoblastic cells showed one case with moderate (2+) and four with intense (3+) staining patterns. Regarding the percentage of positive cells, all cases were listed in category 4 (76 - 100%). The immunoreactive scores ranged between 8 and 12 (median 10) (Figure 4).

A highly significant difference was found regarding Bcl-2 expression (weak vs intense) in cases with Down's syndrome as compared to those of spontaneous abortions ($p < 0.0001$; Fisher's exact test).

Discussion

There are few specific findings that truly characterize a placenta with trisomy but many types of abnormalities have been found sporadically. For example, the incidence of a single umbilical artery (SUA) is higher than normal. Hecht [12] reported five cases of trisomy 18 and found that the associated placentas were unusually markedly increased in fibrin deposits, syncytial knotting, and infracts. Matayoshi *et al.* reported similar findings [13]. Rochelson and colleagues [14] studied the placentas of 18 trisomic fetuses with quantitative morphometry using appropriate controls. Doppler analysis of umbilical arterial blood flow was performed in ten of them. They found that the placentas had a "significant reduction in small muscular artery count and small muscular artery/villus ratio". A quantitative study of normal and chromosomally abnormal placentas between 8 and 13 weeks' gestation was reported by Kuhlmann *et al.* [15]. They found no differences in fetal and placental weights but a significant decrease in small muscular arteries and total vessel counts was seen in the aneuploids. Aside from smaller size, we noted an increase in syncytial knots associated with trisomy 18, increased cellularity of villous stroma, and vascular abnormalities. The last were either old occlusions or fresh thromboses of surface and umbilical cord vessels. Villitis of unknown etiology was found to be spurious. In one case of trisomy 18 associated with many abnormalities of fetal development, the small placenta had numerous cysts scattered throughout. They were composed of large villi with cisternae. Although this appearance was different from that of the partial moles in triploidy, this morphology certainly is not characteristic for, or diagnostic of, trisomy 18. With trisomy 13, the placenta is also frequently dysmature. We have the impression that the villi have deficient capillarization. Saller *et al.* explored the relation of SUA to chromosome errors [16] in 109 chromosomally abnormal pregnancies, with 53 cords identified. Six single umbilical arteries were found (two of 9 trisomy 18; two of 6 with trisomy 13; two of other cytogenetic errors). Quantitative studies, especially combined with assessment of SUA, were not done. Kouvalainen and Østerlund [17] found a somewhat enlarged placental weight in trisomy 21 pregnancies and suggested that it may "be a reflection of an immunological reaction of the mother against her incompatible fetus". Qureshi *et al.* [18] found an increased number of irregular villi (dysmature villi) and some villous hypovascularity in trisomy 21 placentas.

Although Down's syndrome (Trisomy 21) is not accompanied by characteristic pathological changes in the placenta, our study in the series of DS, demonstrated in the placental trophoblast of the chorionic villi (cytotrophoblast and syncytiotrophoblast), a weak expression of the Bcl-2 proto-oncogene, while in the cases of spontaneous abortions an intense Bcl-2 staining was noted ($p < 0.0001$). In the latter cases, Bcl-2 staining pattern in the cytotrophoblast, which predominates at the 12th week (median immunoreactive score 10.5) and 15th week (MIS 10), was as intense as in the syncytiotrophoblast, which predominates at the 22nd week (MIS 10). In contrast, in the cases of DS, trophoblastic cells showed lower values of Bcl-2 expression (5 - 6.5 - 6.5) during the respective periods of gestational age. Results such as, the cyclic variation of Bcl-2 expression in both the glandular and the stromal components of the uterus, and in mammary duct cells, lead to the conclusion that Bcl-2 appears to be expressed in a selected group of terminally differentiated cells which respond to hormonal stimulation.

The differential expression of Bcl-2 protein in the trophoblast between Down's syndrome and spontaneous abortion appears to be due to a possible role of hormones in the regulation of Bcl-2 gene expression.

Our results confirm that Bcl-2 is variably expressed in the chorionic villi of the placental trophoblast. The staining effect in the cytotrophoblast at the 12th and 15th weeks was similar to that in the syncytiotrophoblast at the 22nd week. The syncytiotrophoblast arises from the cytotrophoblast and has numerous important endocrine capacities. Therefore, Bcl-2 theoretically could mediate trophic effects operating as part of a currently uncharacterized endocrine feedback loop. The trophoblast also constitutes the interface between the fetal and maternal circulations; therefore, Bcl-2 protein might save this fetal tissue from death induced by the maternal immune system.

Bcl-2 protein can also save hormone-dependent tissues, such as the endometrium where cyclic changes may, at least in part, be mediated by apoptosis.

In summary, our results lead to the hypothesis that Bcl-2 gene expression is associated with activated cells during the differentiation process and needs protection from apoptosis. This process might be under hormonal control. Our results, though preliminary, also suggest that weak Bcl-2 immunostaining can be of value in the approach to Down's syndrome. These findings therefore suggest that the Bcl-2 gene may have a pivotal role in cell development, maturation, and terminal differentiation, and is worthy of further investigation in this direction.

Acknowledgment

To technician Bachtsevanidou Agorasti for her contribution.

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Address reprint requests to:
N. PAPADOPOULOS, M.D.
Ass't Professor
Dept. of Histology-Embryology
Dragana 68100 Alexandroupolis
(Greece)