Bcl-2 and Bax immunoreactivity in placentas from pregnancies complicated with intrauterine growth restriction and hypertension

Abstract

Because placental apoptosis associated protein imbalance is thought to contribute to the pathogenesis of intrauterine growth restriction (IUGR) and preeclampsia (PE), morphological features and expression of Bcl-2 and Bax were studied on samples from 10 human placentas from pregnancies complicated with IUGR and PE and 10 placentas from uncomplicated term pregnancies.

In 9/10 placentas from the IUGR/PE group, the findings were consistent with hypoxic damage and in 1/10 placentas chronic villitis was found. In both groups Bax showed strong diffuse expression (3+) in amnionic epithelium, blood vessel wall and endothelium in the chorionic plate and placental villi and the villous stroma in all placentas. The extravillous trophoblast (EVT) in the basal decidua showed 3+ Bax expression in 8/10 placentas and 2+ expression in 2/10 placentas in both groups. In the IUGR/PE group the Bax expression in both cyto (CT) and syncytiotrophoblast (ST), was 3+ in 8/10 placentas, and 2+ in 2/10 placentas. In the control group Bax expression was 3+ in both CT and ST in all 10 placentas. The 3+ Bcl-2 expression in both groups was found only in CT and ST. The syncytial knots in both groups showed 3+ positivity for Bax and Bcl-2. These findings do not confirm other authors’ findings about the increased expression of proapoptotic factors in the third trimester placentas with IUGR/PE, and the conclusion is that for the pathogenesis of IUGR and PE, the attention should be shifted to inappropriate apoptosis during the process of implantation.

INTRODUCTION

For the appropriate placenta and development of the placenta the extravillous trophoblast derived from the villi that anchor the developing blastocyst to the underlying decidua plays the most important role. It is the conversion of spiral arterioles into the uteroplacental blood vessels, resulting in the development of a high flow, low resistance vessels able to perfuse the intervillous space. At the surface of the placental villi, the syncytium is formed from underlying mitotically active mononucleated cytotrophoblasts that differentiate and undergo fusion. The anatomical integrity of the syncytiotrophoblast is critical, as a variety of receptors and enzymes are strategically positioned on the maternal blood facing membrane and regulate maternal–fetal exchange (1). The villous
trophoblast bilayer is delimited from the villous stroma which contains branching fetal blood vessels in a connective tissue matrix by a basement membrane. Placental function is critical at all stages of pregnancy, but nutrient transport demands on villous trophoblasts are highest as the fetus triples in weight during the third trimester of gestation (2). In preeclampsia (PE) a characteristic change of the spiral arteries, firstly described and named „acute atherosis“ can be seen in the basal decidua layer, as well as in the free placental membranes (3). Acute atherosis develops only in the spiral arteries that have not undergone physiologic changes, and the reason for this incomplete change still remains a mystery. Some authors have found a reduction in the extent of trophoblast invasion in severe preeclampsia, both in the spiral arteries and the myometrium (4). Poor trophoblast invasion and remodeling of uterine spiral arteries have been suggested to lead to hypoperfusion, hypoxia, reperfusion injury, oxidative stress and signs of villous tree maldevelopment in the second half of the pregnancy. Hypoxic environment in cases of impaired uteroplacental blood flow, causes necrosis of superficial, syncytiotrophoblastic layer with subsequent discontinuities. In normal circumstances, within the villous trophoblast, proliferation is restricted to the cytotrophoblast, and apoptosis is almost exclusively localised to the syncytiotrophoblast (5). Bax, one of the proapoptotic members of the Bcl-2 family, is present in the villous trophoblast in the first and third trimester. In the first trimester, it is localized to the cytoplasm of the cytotrophoblast, but in the third trimester it is expressed in the syncytiotrophoblast (6-8). The levels of apoptosis within tertiary villi increase with gestation, and are the greatest over 40 weeks of gestation (9). Insults that result in villous trophoblast injury may not be accompanied by a compensatory increase in cytotrophoblast proliferation and differentiation (10, 11). Because of the extreme importance of apoptosis regulation, cultured trophoblasts exposed to hypoxia show a marked upregulation of p53 activity, enhanced expression of the pro-apoptotic Mtd-1 and decreased expression of the anti-apoptotic Bcl-2, all of which promote apoptosis (8, 12, 13). In contrast to hypoxia alone, hypoxia-reoxygenation results in more marked apoptosis regulated by other proteins such as the increased expression of the pro-apoptotic Bax and Bak (13-15). Expression of Bax is often associated with areas of trophoblast damage, or degeneration, such as fibrinoid deposits and syncitial knots (16, 17).

With all the data from the literature, we hypothesized that the expression of proapoptotic Bax protein will be enhanced in the tissue of placentas from the pregnancies complicated with PE and intrauterine growth restriction (IUGR), in comparison to the placentas from uncomplicated term pregnancies. We also expected the expression of anti-apoptotic Bcl-2 protein to be strong in the areas of villous trophoblast damage, and in the areas of fibrinoid deposition. This study is a pilot study of a larger study with the same hypotheses and aim which was to assess the expression of Bax and Bcl-2 proteins in different compartments of the placentas from the pregnancies complicated with IUGR and PE, to compare the results with the expression of Bax and Bcl-2 in the corresponding compartments of the placentas with uncomplicated term pregnancies.

MATERIALS AND METHODS

The material consisted of 10 samples of human placental tissue from the pregnancies complicated with IUGR and PE confirmed by serial clinical follow up. The control group consisted of 10 samples of human placental tissue from the uncomplicated term pregnancies. The tissue in both groups was retrieved from the archive of the Clinical Department of Pathology „Ljudevit Jurak“, Clinical Hospital Center „Sestre Milosrdnice“ in Zagreb, where they were collected during the scientific project of the Ministry of science, education and sport of the Republic of Croatia, No. 108-1081870-1940. The samples contained full thickness of the placenta, from the chorionic plate membranes to the basal decidua, that were taken from the unfixed placental tissue during routine pathological examination. The samples were routinely fixed in formalin and embedded in paraffin. Three sections of every sample were cut at 5 µm; one was stained routinely with hematoxylin-eosin (H-E) and examined by light microscopy to assess the morphology; the other two were analyzed after immunohistochemical staining for Bax (polyclonal rabbit anti-human, Clone A5333, Dako Denmark) and Bcl-2 (monoclonal mouse anti-human, Clone 124, Code M0887, Dako, Denmark), respectively. For immunohistochemical analysis the 5 µm thick sections of the placental tissue were mounted on a silanized slide, and left to dry for 24 h. The section were than deparaffinized in xylene, rehydrated through a graded series of alcohol and washed in phosphate buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were treated with Catalyzed Signal Amplification (CSA) System II for use with mouse primary antibod (Code K1497, Dako, Denmark). The CSA System II was adapted for the detection of rabbit polyclonal antibodies by replacing the anti mouse link provided in the kit with the CSA II Rabbit Link (Code 1501, Dako, Denmark). Immunohistochemical staining intensity was evaluated semi-quantitatively as: negative (-), weakly positive (1+) if positive in solitary cells, up to 50% of cells; moderately positive (2+) if strongly positive in up to 50% of cells, moderately positive in > 50% of cells and strongly positive (3+) if strongly positive in > 50% of cells. The immunoreactivity was assessed in different parts of the placenta: extravillous trophoblast (EVT) in the basal decidua, villous cytotrophoblast (CT) and syncytiotrophoblast (ST), the villous stromal cells, the blood vessels of the chorionic plate and in the amnionic
epithelium of the chorionic plate. For the analysis of the results, descriptive methods were used.

**RESULTS**

The mean gestational age of women in group with IUGR/PE was 36 weeks (range 31 – 39 weeks), and in the control group it was 39 weeks (range 37 – 40 weeks). The mean placental weight in the group with IUGR/PE was 330 g (range 250 – 380 g), and in the control group 438 g (range 390 – 520 g). Because of the fact that pregnancies complicated with PE and IUGR frequently terminate earlier, either spontaneously, or because of the threatening asphyxia of the fetus, there was no point in calculating statistical significance of the difference.

Grossly, one placenta in the control group was succenturiate, but on microscopic examination showed normally developed, term villi. On light microscopy histopathological examination of H-E stained placental tissue, fetal membranes and the umbilical cord showed normal morphology in all 10 placentas from IUGR/PE group as well as in the control group. Out of 10 placenta in the IUGR/PE group, none showed completely normal histological features. In 9/10 placentas the findings were consistent with hypoxic damage, while in 1/10 placentas a diffuse chronic villitis of unknown etiology (VUE) was found. In the control group of placentas, 1/10 showed a solitary, peripherally localized chronic infarct. The histopathological findings of the placental tissue stained with H-E are shown in Table 1.

Immunoreactivity for Bax in both, group with IUGR/PE and the control group showed the same pattern in all 10 placentas in each group: strong diffuse expression (3+) in amnionic epithelium (Figure 1) blood vessel wall and endothelium in the chorionic plate and the placental villi (Figure 2), and the villous stroma. In the EVT in the basal decidua strong expression (3+) was found in 8/10 placentas in both groups, while the expression was graded as moderate (2+) in 2/10 placentas in both groups (Figure 3). The Bax expression was strong and diffuse (3+) in both CT and ST in the IUGR/PE group in 8/10 placentas, and moderate (2+) in 2/10 placentas in this group (Figure 4). In the control group Bax expression was strong and diffuse (3+) in both CT and ST in all 10/10 placentas (Figure 5). The syncytial knots in both groups showed strong (3+) positivity for Bax (Figure 6). The results of Bax expression are summarized in Table 2.

The Bcl-2 expression in both groups was found only in CT and ST, and estimated as strong (3+) in both analyzed groups (Figure 7). All other elements were negative for Bcl-2 (Table 3).
In PE and IUGR, the placenta is frequently small (weight under the 10th percentile for gestational age, feto-placental ratio inappropriate for gestational age), with gross findings of multiple infarcts, and (especially in PE) 3-5 times more frequent findings of retroplacental hematoma. Histological findings may be very different, but mostly point out towards the inappropriate uteroplacental blood flow, such as the thickened cytotrophoblastic layer, very numerous syncitial knots and chorangiosis (18). Due to the development of a high pressure placental blood supply, the developing villous tree can be damaged, showing changes in placental structure (19, 20). In placentas from pregnancies complicated by IUGR these sites of injury and repair are covered with fibrin containing deposits, that can be found in every placenta, but are observed with increased frequency in placentas with IUGR. The fibrin matrix serves as a scaffold for trophoblast to re-epithelialize the villous surface. Syncytial knots, that are also observed in much greater quantity in placentas with PE, are clusters of apoptotic syncytial nuclei that bulge from the surface into the intervillous space. The underlying cytotrophoblastic population provides a source for new syncytium during normal epithelial turnover and at sites of syncytial re-epithelialization of fibrin on the surface of villi, resulting with thickened cytotrophoblastic layer seen on light microscopy (18).
Programmed cell death by apoptosis and its associated regulatory mechanisms are intimately involved in placental homeostasis, growth and remodeling with the apoptotic rates that increase progressively during normal pregnancy as part of normal placental development (21). Previous studies showed that Bcl-2 was generally expressed at low levels during the entire gestational period, while Bax was low during the first trimester and increased towards the end of gestation. In accordance with the change of ratio of these two molecules, the increase of apoptotic cells was observable in the third trimester (22). In their study of Bcl-2 and Bax expression in preterm, term and postterm placentas Daher et al. found the same pattern of immunostaining for Bcl-2 and Bax in all samples, but reactivity for Bax was higher in preterm and postterm placentas, whether the reactivity for Bcl-2 decreased in preterm placentas. This reactivity pattern resulted in the higher Bax/Bcl-2 ratio in both pre-term and post-term placental samples compared with term placentas (23). All these data indicate that Bcl-2 and Bax are temporally regulated during placental development and that the different expression of the above mentioned genes is at least in part responsible for the delicate balance between cell proliferation and programmed cell death in the human placenta during pregnancy.

In the present study, the analysis of Bax showed strong or, in the minority of cases, moderate expression in CT, ST, EVT, stromal cells of the villi, and endothelial cells in both analyzed groups. These findings confirm the findings of Cobelli et al. (24).

Although many molecules are associated with the induction and prevention of apoptosis in different models one of them is a proapoptotic factor Bax and the same authors also observed an increase of Bax expression in all placental compartments in preeclampsia and diabetes, compared to term placentas from uncomplicated pregnancies (24). Several studies demonstrated that apoptosis increases in pregnancies complicated by some pathologies such as preeclampsia, fetal growth restriction and diabetes (21, 25-27). Other authors’ observations were similar, not only by means of immunohistochemistry alone, but also with combined methods and TUNEL (terminal deoxyribonucleotidyltransferase-mediated dUTP and-labelling) (16). The etiology of IUGR and PE is still unclear, however placental dysfunction is considered a common underlying cause in both conditions. The role of apoptosis in the development of placental pathology of these conditions has yet to be explained. The theory that the placentas in these pregnancy disorders display altered cell kinetics is corroborated by the findings of an increase of Bax expression is observed in all the placental compartments, especially in preeclampsia (28). In both IUGR and PE, apoptosis may disrupt the cytotrophoblast turnover that is, especially in PE increased, commencing with increased prolifera-

tion of cytotrophoblast, that could produce increased end stages of apoptosis in the syncytioblast (25). Han et al. demonstrated that caspase-10 and death receptor 3 (DR-3) were upregulated in placenta from preeclamptic patients, suggesting that placental apoptosis and altered gene expression in the trophoblast may influence the pathogenesis of preeclampsia (27).

The results of this study did not confirm the above mentioned findings, because the expression of Bax was practically of the same intensity in the group of placentas with IUGR/PE and the control group in all placental compartments. Perhaps the studied group was too small, perhaps the immunoreagents was not properly chosen and perhaps in this group there was just no difference. Although some authors found that the expression of Bax is often associated with fibrinoid deposits and syncitial knots, in this study no difference was found between the expression of Bax in fibrinoid deposits in the investigated placentas and the control group (16, 17). As to the expression of Bcl-2, many factors capable of regulating apoptosis are present in the villous trophoblast, but it seems that their expression change with pregnancy progression. The syncytiotrophoblast is protected against unwanted apoptosis by expression of Bcl-2 (and some other anti-apoptotic factors). In this study, the expression of Bcl-2 was restricted to villous trophoblast, where it was found to be mostly strong, and in several cases (in the IUGR/PE group) moderate. It was also strong in the syncytiotrophoblast, in both analyzed groups, but the expression of Bax was found to be strong in both analyzed groups as well.

Recently, it has been suggested that vascular remodeling that occurs during placentation may be indirectly controlled by intravascular trophoblast that stimulates endothelial cells to secrete chemokines. These chemokines attract decidual leukocytes, particularly uterine natural killer cells and macrophages, leading to vascular smooth muscle cell apoptosis (29). A suggested mechanism for endothelial cell destruction is via the Fas/FasL system, which is present on endothelial and vascular smooth muscle cells of the uterine spiral arteries (30). In PE and IUGR, there may be a reduction in the number of trophoblast cells within the spiral arteries, which has been associated with increased apoptosis and a reduced luminal size (30, 31). Many authors have found significant increases in placental apoptosis, which may be the underlying cause in the pathophysiology of preeclampsia and IUGR, but this was not confirmed in this study. The reason for this discrepancy cannot be found. However, there is a growing body of evidence that suggests that abnormal apoptosis has important effects at the very beginning of the placental development, namely the physiological conversion of spiral arteries to uteroplacental vessels. In our opinion, this is the real cause of IUGR and/or PE, and the attention has to be shifted from the analysis of apoptosis in the third trimester or term pla-
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