

Expression of the retinoblastoma-related p107 and Rb2/p130 genes in human placenta: an immunohistochemical study

I. Cavallotti¹, L. De Luca¹, A. D'Aponte¹, M. De Falco²,
F. Acanfora¹, M.L. Visciano¹, L. Gualdiero¹, B. De Luca¹, A. Baldi³ and A. De Luca¹

¹Institute of Topographical Anatomy, School of Medicine, Second University of Naples, Naples, Italy,

²Department of Evolutive and Comparative Biology, University "Federico II", Naples, Italy and

³Laboratory of Cell Metabolism and Pharmacokinetics, Center for Experimental Research, Regina Elena Institute, Rome, Italy

Summary. It has been proposed that tumor suppressor genes may have a role in the mechanisms of proliferation and differentiation during human placental development. The Retinoblastoma gene family is a well known family of tumor suppressor genes. Many studies have pointed out a role of this family not only in cell cycle progression, but also during development and differentiation. On the light of these observations we have investigated the immunohistochemical expression pattern of the Retinoblastoma family members, p107 and Rb2/p130 in human placenta samples in first trimester and full-term placental sections. p107 and pRb2/p130 showed the most abundant expression levels during the first trimester of gestation and progressively declined to being barely detectable in the placenta by late gestation. These results indicate that the expression of the above genes is modulated during placental development and suggest a mechanism for controlling trophoblast proliferation.

Key words: Rb2/p130, p107, Retinoblastoma, Immunohistochemistry, Placenta

Introduction

The retinoblastoma (Rb) gene family contains three members: the Rb gene, which is the prototypic tumor suppressor gene, and two related genes, p107 and Rb2/p130. These genes share a high percentage of sequence homology especially in their pocket region that has a distinctive steric conformation responsible for most of the functional interactions characterizing the activity of Rb and Rb-related genes (for a review De Luca et al., 1997). Functionally, all the Rb family members show cell growth suppressive properties and

can bind to specific members of the E2F family of transcription factors (Claudio et al., 1994). In addition, pRb, p107 and pRb2/p130 are associated with various cyclins in different phases of the cell cycle, and are phosphorylated in a cell cycle dependent-manner (Baldi et al., 1995; Claudio et al., 1996). These activities and interactions allow the Rb family genes to serve as signal transducers linking the cell cycle and transcriptional machinery. Moreover, all the Rb family members, because of their ability to negatively regulate cell proliferation and to their precise relationship with the cell cycle checkpoints, have often been found to be involved in differentiative processes (for a review Paggi et al., 1996).

The placenta allows the exchange of metabolic and gaseous products between the maternal and fetal blood flow without mixing with each other. It has a discoid aspect at the end of the development, showing a fetal as well as a maternal surface. The placenta undergoes extensive proliferation during gestation and exhibits invasive growth comparable to that of malignant tumor (Ohlsson et al., 1993). In particular, in the first trimester of human pregnancy, the cytotrophoblast proliferates and invades deep into the maternal decidua under strict temporal and spatial control. Around the 13th day the chorionic villi begin to appear in the shape of syncytial trabeculae separated by lacunae. From the second to the fourth month the villi ramify and are still covered by syncytium trophoblast on the surface and by cytotrophoblast in depth. After the fourth month the villus has become very arborized and cytotrophoblast has almost completely disappeared.

It has been hypothesized that a decreased proliferation, occurring as pregnancy proceeds into the second and third trimester (Hamilton et al., 1962), is one method of controlling trophoblast cell invasion. Taking into account the roles played by retinoblastoma gene family members in cell proliferation and development, we decided to investigate the immunohistochemical expression of p107 and Rb2/p130 in a series of human placenta in first trimester and full-term placental

Offprint request to: Dr. Antonio De Luca, Institute of Topographical Anatomy, School of Medicine, 2nd University of Naples, Via L. Armanni 5, 80138 Naples, Italy. Fax: +39 (081) 458225. e-mail: antonio.deluca@unina2.it.

sections.

Materials and methods

Normal tissues

Placentas from the first and last trimester were collected from therapeutic abortion or immediately after spontaneous delivery. We evaluated 50 samples, 25 from first and 25 from the last trimester. The tissues were formalin-fixed and paraffin-embedded. Representative sections of each specimen were stained with hematoxylin-eosin and examined by a pathologist to confirm the histological preservation of the microanatomic structure.

Antibodies

The rabbit polyclonal immune serum against Rb2/p130 (ADL1) was produced by immunizing rabbits with a peptide corresponding with the carboxy terminus of the protein. The polyclonal antibody against p107 (ADL2) was produced by immunizing rabbits with a bacterially expressed glutathione S-transferase (GST) p107 fusion protein. Expression of the fusion protein was performed as previously reported (Frangioni and Neel, 1993). The suitability of both antibodies in immunohistochemical assays has already been described (Baldi et al., 1996, 1997).

Immunohistochemistry

Sections from each specimen were cut at 3-5 μ m, mounted on glass and dried overnight at 37 °C. All sections, were then deparaffinized in xylene, rehydrated through a graded alcohol series and washed in PBS. This buffer was used for all subsequent washes and for dilution of the antibodies. Tissue sections were sequentially quenched in 0.5% hydrogen peroxide and blocked with diluted 10% normal goat anti-rabbit serum

(Vector Laboratories). Slides were incubated for 1 hour with the rabbit polyclonal immune serum ADL1 (dilution 1:1000) and ADL2 (dilution 1:500). Then, slides were incubated with diluted goat anti-rabbit biotinylated antibody (Vector Laboratories) for 30 minutes at room temperature. Finally, all the slides were processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine was used as the final chromogen. Negative controls for each tissue section were prepared by substituting the primary antibody with the respective preimmune serum. All samples were processed under the same conditions. Three observers evaluated the staining pattern of the protein separately and scored it for the percentage of positive nuclei: score 0 (less than 1% of positive cells), score 1 (from 1% to 10% of positive cells); score 2 (from 10% to 20% of positive cells); score 3 (more than 20% of positive cells). The level of concordance, expressed as the percentage of agreement between the observers, was 92%. In the remaining specimens the opinions of the two investigators in agreement were taken in considerations.

Results

We investigated the expression pattern of the two retinoblastoma-related gene p107 and Rb2/p130 in human placenta throughout the entire gestation. Considering the few case that we were able to obtain in the middle gestation, our attention was focused only on the first and last trimester. Placentae from the first and last trimester were collected from therapeutic abortion or immediately after spontaneous delivery. This study was conducted in two components of the villous: the inner proliferative compartment (cytotrophoblast) and the differentiated cell layer (syncytiotrophoblast).

p107 and Rb2/p130 were both found expressed in the villous in the first trimester, then steadily decreased. In Fig. 1A,B two representative stainings for p107 and Rb2/p130 are depicted. The overall expression of both

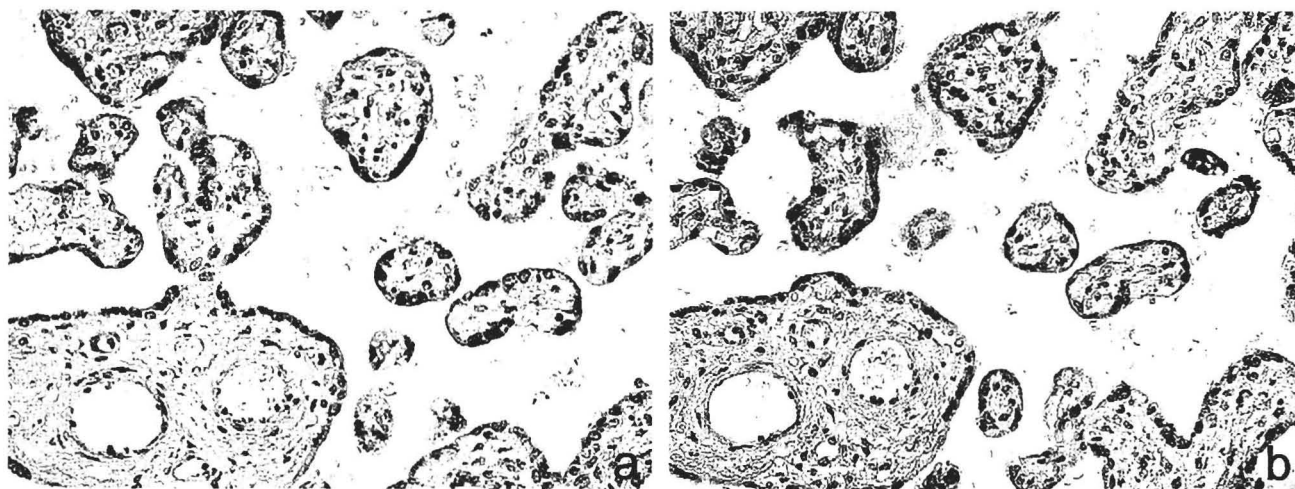


Fig. 1. Representative pictures of serial sections of first trimester of placenta tissue immunostained for p107 (A) and pRb2/p130 (B). x 250

proteins was found higher in the syncytiotrophoblast than in the cytotrophoblast. In the last trimester of gestation either p107 or pRb2/p130 steadily decreased in both compartments to a low level. In particular pRb2/p130 decreased almost to an undetectable level in the syncytiotrophoblast. In figure 2 the different expression levels of the two proteins vs weeks of pregnancy are depicted.

Decidual tissue during the first trimester revealed a diffuse immunoreactivity for both proteins, being the expression of p107 slightly higher compared with Rb2/p130. At later gestational ages the majority of decidual cells remained negative for both proteins (data not shown).

Discussion

The implantation of the villi in the uterine wall is an aggressive event that in some way mimics an invasive tumor. For this reason the term "pseudo-malignancy" has been used to describe the properties of the early human placenta (Ohlsson et al., 1993). In a normal implantation the invasion of the uterus is controlled in a spatial and temporal limit of migration and the uterus limits this implantation. The molecular mechanisms responsible for controlling trophoblast proliferation are still unclear.

Several previous works have looked at the expression of oncogenes and tumor suppressor genes, as well as of anti-apoptotic proteins during development of human placenta (Pfeifer-Ohlsson et al., 1984; Roncalli et al., 1994; Marzusch et al., 1995; Quenby et al., 1998; Fulop et al., 1998). It is generally concluded that the simultaneous activity of several proteins involved in cell proliferation and/or apoptosis during trophoblast differentiation plays an important role both in the regulation of cell invasion and population expansion, especially during the first trimester (Levy and Nelson, 2000).

In addition to the cell cycle, the RB family regulates a wide spectrum of complex biological phenomena, such as differentiation, embryonic development and apoptosis (De Luca et al., 1996). Considering the fundamental role of pRb in differentiation and development, it is conceivable that pRb2/p130 and p107 may serve a similar function.

Today the roles played by retinoblastoma-related p107 and Rb2/p130 during proliferation and differentiation are well known. In particular, it has been described, at least for Rb2/p130, that its protein expression level is modulated during terminal differentiation (Pertile et al., 1995; Baldi et al., 1997). Drawing from this background, we decided to look at the

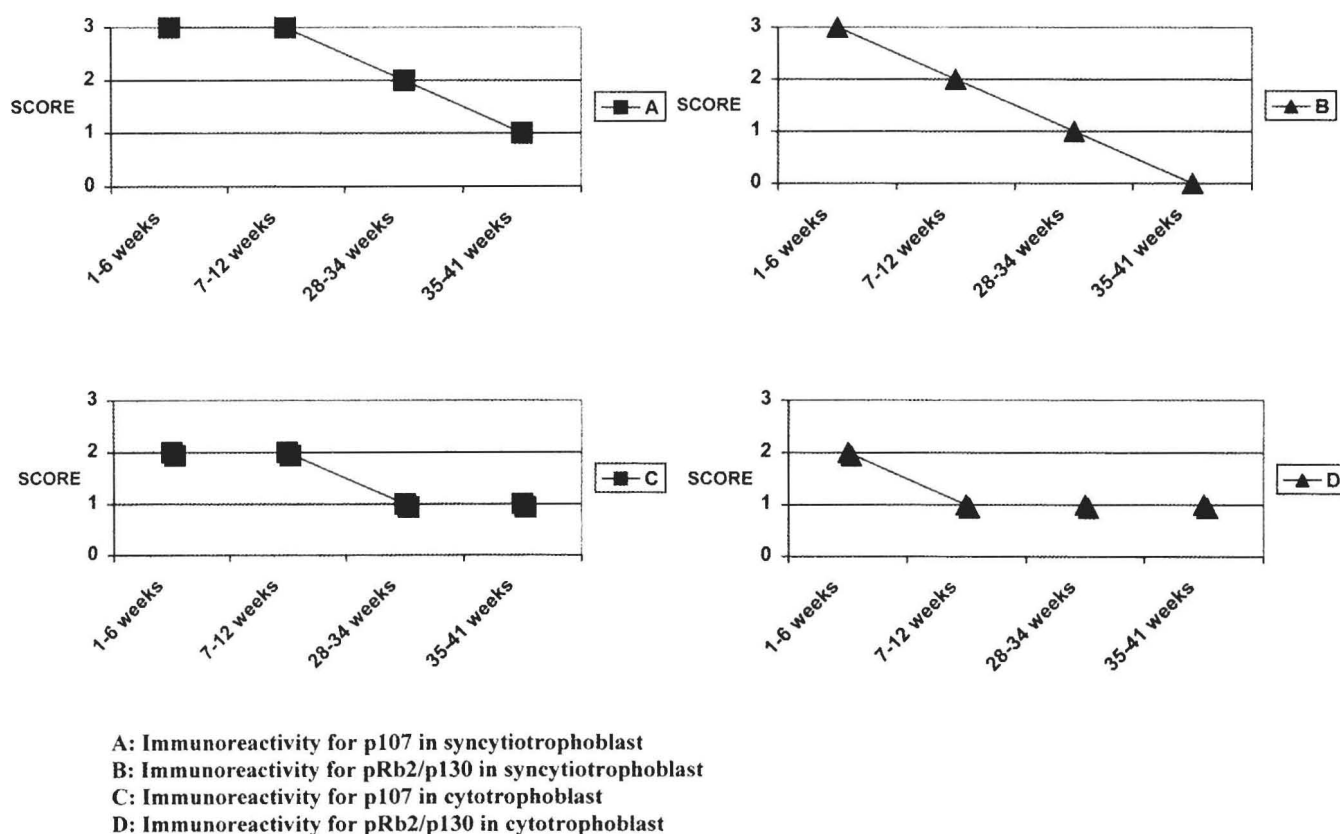


Fig. 2. Gestational time course of syncytiotrophoblast and cytotrophoblastic immunoreactivity for the different antigens under study. Ordinate: immunoreactivity scored as described in materials and methods. Abscissa: Weeks of gestations. **A.** Immunoreactivity for p107 in syncytiotrophoblast. **B.** Immunoreactivity for pRb2/p130 in syncytiotrophoblast. **C.** Immunoreactivity for p107 in cytotrophoblast. **D.** Immunoreactivity for pRb2/p130 in cytotrophoblast.

immunohistochemical expression of these two proteins in developing and term human trophoblast. Our data indicate that the expression of these two genes is spatio-temporal regulated during the entire placental development. Rb2/p130 and p107 are expressed in the proliferative trophoblastic compartment (cytotrophoblast) since the beginning of the gestation and their expression declines with the decrease of the proliferation rate during the gestation. In the syncytiotrophoblastic cells the immunoreactivity for pRb2/p130 and p107 reaches a peak at the end of the first trimester of gestation. When the syncytiotrophoblast, in the mid-gestation, switches its function from an invasive to nutritional one, the expression of pRb2/p130 and p107 drops dramatically. In particular pRb2/p130 drops almost to an undetectable level.

It is interesting to note that the expression pattern of p107 and Rb2/p130 differs from that of the Rb gene. In fact, while in the cytotrophoblast the three proteins behave similarly, in the syncytiotrophoblast they have a consistent by different expression pattern. Rb is always expressed at low to undetectable levels (Roncalli et al., 1994; Quenby et al., 1998), while p107 and Rb2/p130 reach a peak of expression at the end of the first trimester of gestation and then decrease to almost undetectable levels.

These results indicate that p107 and Rb2/p130 enter in the complex molecular pathway controlling cell proliferation and population expansion during placental development. In particular, they may play a role in the decreased proliferation occurring as pregnancy proceeds into the second and third trimester, as it has already been suggested for other tumor suppressors such as p53 and Rb (Roncalli et al., 1994; Marzusch et al., 1995). Moreover, the peculiar expression pattern of these two proteins in the syncytiotrophoblast, suggest a possible role in the switch of function from an invasive to a nutritional one of human placenta.

At today is known that the three members shows different levels of expression of these proteins in order to the maintenance of the terminal differentiation status (Pertile et al., 1995; Baldi et al., 1997). However, few informations are known on the phosphorylation status of these proteins in differentiated tissues. In the future, evaluate the phosphorylation status of these proteins throughout gestation will be important in order to understand if any correlation occur between the expression and the functional status of the three members of the Rb gene family.

Acknowledgements. The authors thank Mr. Tullio Battista (Regina Elena Institute, Rome, Italy) for his excellent technical assistance. A.B. is recipient of a FIRC fellowship.

References

- Baldi A., De Luca A., Claudio P.P., Baldi F., Giordano G.G., Tommasino M., Paggi M.G. and Giordano A. (1995). The Rb2/p130 gene product is a nuclear protein whose phosphorylation is cell cycle regulated. *J. Cell. Biochem.* 59, 402-408.
- Baldi A., Esposito V., De Luca A., Howard C.M., Mazzarella G., Baldi F., Caputi M. and Giordano A. (1996). Differential expression of the retinoblastoma gene family members, Rb, p107 and Rb2/p130 in lung cancer. *Clin. Cancer Res.* 2, 1239-1245.
- Baldi A., Esposito V., De Luca A., Fu Y., Meoli I., Giordano G.G., Caputi M., Baldi F. and Giordano A. (1997). Differential expression of Rb2/p130 and p107 in normal human tissues and in primary lung cancer. *Clin. Cancer Res.* 3, 1691-1697.
- Claudio P.P., Howard C.M., Baldi A., De Luca A., Fu Y., Condorelli G., Sun Y., Colburn N., Calabretta B. and Giordano A. (1994). P130/Rb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. *Cancer Res.* 54, 5556-5560.
- Claudio P.P., De Luca A., Howard C.M., Baldi A., Firpo E.J., Paggi M.G. and Giordano A. (1996). Functional analysis of pRb2/p130 interaction with cyclins. *Cancer Res.* 56, 2003-2008.
- De Luca A., Esposito V., Baldi A. and Giordano A. (1996). The retinoblastoma gene family and its role in proliferation, differentiation and development. *Histol. Histopathol.* 11, 1029-1034.
- Frangioni J.V. and Neel B.G. (1993). Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal. Biochem.* 210, 179-187.
- Fulop V., Mok S.C., Genest D.R., Gati I., Doszpod J. and Berkowitz R.S. (1998). C-myc, c-erbB-2, c-fms and bcl-2 oncoproteins. Expression in normal placenta, partial and complete mole, and choriocarcinoma. *J. Reprod. Med.* 43, 119-127.
- Hamilton W.J., Boyd J.D. and Mossman H.W. (1962). Human embryology, prenatal development form and function. Heffer and Sons. Cambridge, UK.
- Levy R. and Nelson D.M. (2000). To be, or not to be, that is the question. Apoptosis in human trophoblast. *Placenta* 21, 1-13.
- Marzusch K., Ruck P., Horny H.P., Dietl J. and Kaiserling E. (1995). Expression of the p53 tumor suppressor gene in human placenta: an immunohistochemical study. *Placenta* 16, 101-104.
- Ohlsson R., Glaser A. and Holmgren L. (1993). The molecular biology of placental development. In: *The human placenta*. Redman, C.W.G., Sargent I.L. and Starkey P.M. (eds). Blackwell Scientific Publications. Oxford, UK. pp 598.
- Paggi M.G., Baldi A., Bonetto F. and Giordano A. (1996). Retinoblastoma protein family in cell cycle and a cancer: a review. *J. Cell. Biochem.* 62, 418-430.
- Pertile P., Baldi A., De Luca A., Bagella L., Virgilio L., Pisano M.M. and Giordano A. (1995). Molecular cloning, expression, and developmental characterization of the murine retinoblastoma-related gene Rb2/p130. *Cell Growth Differ.* 6, 1659-1664.
- Pfeifer-Ohlsson S., Goustin A.S., Rydner J., Wahlstrom T., Bjersing L., Stehelin D. and Ohlsson R. (1984). Spatial and temporal patterns of cellular myc oncogene expression in developing human placenta: implications for embryonic cell proliferation. *Cell* 38, 585-596.
- Quenby S., Brazeau C., Drakeley A., Lewis-Jones D.I. and Vince G. (1998). Oncogene and tumor suppressor gene products during trophoblast differentiation in the first trimester. *Mol. Hum. Reprod.* 4, 477-481.
- Roncalli M., Bulfamante G., Viale G., Springall D.R., Alafano R., Comi A., Maggioni M., Polak J.M. and Coggi G. (1994). C-myc and tumor suppressor gene product expression in developing and term human trophoblast. *Placenta* 15, 399-409.

Accepted June 8, 2001