Acta Histochemica 117 (2015) 137–147

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

# Determination of PCNA, cyclin D3, p27, p57 and apoptosis rate in normal and dexamethasone-induced intrauterine growth restricted rat placentas

Hakan Er[a,b,1], Nuray Acar[a,1], Dijle Kipmen-Korgun[c], Ciler Celik-Ozenci[a], Ismail Ustunel[a], Mevlut Asar[a], Emin Turkay Korgun[a,*]

[a] Department of Histology and Embryology, Medical Faculty, Akdeniz University, 07070 Antalya, Turkey
[b] Department of Biophysics, Medical Faculty, Akdeniz University, 07070 Antalya, Turkey
[c] Department of Biochemistry, Medical Faculty, Akdeniz University, 07070 Antalya, Turkey

ARTICLE INFO

*Article history:*
Received 26 August 2014
Received in revised form
24 November 2014
Accepted 25 November 2014

*Keywords:*
Apoptosis
Cyclin D3
PCNA
p27
p57
Rat placenta

ABSTRACT

Intrauterine growth restriction (IUGR) is a major clinical problem, which causes perinatal morbidity and mortality. One of the reasons for IUGR is abnormal placentation. In rats, fetal–placental exposure to maternally administered glucocorticoids decreases birth weight and placental weight. Proper placental development depends on the proliferation and differentiation of trophoblasts. Our knowledge about the mitotic regulators that play key roles in synchronizing these events is limited. Also the mechanisms underlying the placental growth inhibitory effects of glucocorticoids have not been elucidated. The aim of this study was to investigate the immunolocalization, mRNA and protein levels of proliferating cell nuclear antigen (PCNA), cyclin D3, p27 and p57 in normal and dexamethasone-induced IUGR Wistar rat placentas by reverse transcriptase polymerase chain reaction (RT-PCR), immunohistochemistry and Western blot. We also compared apoptotic cell numbers at the light microscopic level via terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) and transmission electron microscopy. Glucocorticoid levels were higher in IUGR rats than in control rats after 60 and 120 min of injection. We showed reduced gene and protein expressions of PCNA and cyclin D3 and increased expressions of p27 and p57 in IUGR placentas compared to control placentas. Apoptotic cell number was higher in the placentas of the IUGR group. In brief we found that maternal dexamethasone treatment led to a shift from cell proliferation to apoptosis in IUGR placentas. Dexamethasone induced placental and embryonal abnormalities which could be associated with reduced expressions of PCNA and cyclin D3, increased expressions of p27 and p57 and increased rate of apoptosis in IUGR placentas.


## Introduction

The placenta promotes the delivery of nutrients and oxygen from the maternal circulation to the fetus and is essential for fetal growth. It forms the surface for transport of nutrients and gas exchange and directs blood supply to the uterus (Hemberger and Cross, 2001). Although the architecture of the human and rodent placentas show minor differences, however, their overall structure and the molecular mechanisms of placental development are thought to be very similar. As a result, the rat placenta is increasingly used as a model to investigate the mechanisms of placental development (Rossant and Cross, 2001) and pregnancy-related problems in humans, such as those associated with diabetes, hypertension and IUGR (Vercruysse et al., 2006).

The eukaryotic cell cycle is regulated by the coordinated activity of a family of cyclin-dependent kinases (CDKs). These are positively and negatively regulated by the cyclin and CDK inhibitor (CKI) families, respectively (Sherr and Roberts, 1995; Xiong, 1996).

Based on the timing of their appearance in the cell cycle, cyclins can be divided into two groups, the mitotic cyclins A and B, and the

*Abbreviations:* AEC, 3-amino-9-ethylcarbazole; AI, apoptotic index; CDK, cyclin dependent kinases; CKI, CDK inhibitor; Dex, dexamethasone; ELISA, enzyme-linked immunosorbent assay; IUGR, intrauterine growth restriction; JZ, junctional zone; LZ, labyrinth zone; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcriptase polymerase chain reaction; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling.

* Corresponding author.
*E-mail address:* korgun@akdeniz.edu.tr (E.T. Korgun).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.acthis.2014.11.010
0065-1281/

G1 cyclins of the D and E family (Lew et al., 1991). D-type cyclins (cyclins D1, D2, or D3) promote progression from mid to late G1 via CDK4 and CDK6 activation (Sherr and Roberts, 1995) and are required for S-phase entry, providing a link between the cell cycle, signal transduction and proliferation. Cyclin E-CDK2 complexes are also active during late G1 phase and are implicated in G1/S progression (Koff et al., 1992). Cyclin E, one of the G1 cyclins, is expressed during mid- to late-G1 phase. Kinase activities of cyclin E/CDK2 complexes are at maximum levels before S-phase entry.

Proliferating cell nuclear antigen (PCNA) is a marker for the cell cycle, its expression peaks in late G1 and S phases of the cell cycle (Takahashi and Caviness, 1993). It is an accessory protein that is necessary for deoxyribonucleic acid (DNA) synthesis in mammalian cells (Murphy et al., 1974), whereas lack of this protein prevents cell division (Braun et al., 2007). It is an essential regulator of p21 activity by binding to the carboxy terminus of p21, which then results in the inhibition of PCNA-dependent DNA replication. Proliferating cell nuclear antigen loads onto DNA through the action of replication factor C complex and provides a scaffold for consecutive attachment of various DNA nucleases, DNA polymerases, DNA ligases and others, and is involved in DNA replication, recombination and repair (Goland et al., 1995).

Cell cycle arrest and activation of differentiation processes require inhibition of CDKs by CKIs (Rao et al., 1994; Skapek et al., 1995, 1996). In mammalian cells, there are at least two distinct families of CKIs: the INK4 and the Cip/Kip inhibitors. INK4 family members include p15, p16, p18, and p19. Cip/Kip members include p21, p27, and p57 (el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Polyak et al., 1994; Toyoshima and Hunter, 1994; Lee et al., 1995). Both families play regulatory roles during the G1/S cell cycle checkpoint (Hunter and Pines, 1994). Because of their broader panel of CDKs with which they interact (Harper and Elledge, 1996), the inhibitors of the Cip/Kip family control other checkpoints as well. Also, p27 has the capacity to arrest cells in G2 (Niculescu et al., 1998). p57 (Kip2) inhibits cyclin A- and E-associated CDKs and therefore regulates G1/S transition and completion of S phase (Lee et al., 1995) and is primarily expressed in terminally differentiated cells (Yan et al., 1997).

IUGR is a major cause of perinatal death and neonatal morbidity and mortality. There are numerous causes of IUGR. Glucocorticoid induced IUGR is highly relevant because administration of synthetic glucocorticoids, principally dexamethasone, to women threatened by premature labor is widely used in clinical practice. Fetal growth is directly related to placental growth and development.

Synthetic glucocorticoids, including dexamethasone, have been used for over three decades to mature fetal lungs and prevent respiratory distress syndrome in pregnancies where delivery is likely before 34 weeks (Liggins and Howie, 1972; Trainer, 2002). Glucocorticoids play an essential role in normal fetal development and are important for the maturation of fetal tissues in preparation for extra-uterine life (Fowden et al., 1998). Although glucocorticoids promote lung maturation, these actions are not without negative side effects. Exposure to glucocorticoids retards fetal growth in animal models and in humans (Benediktsson et al., 1993; Seckl, 1994; Gluckman, 2001; Sugden et al., 2001; McDonald et al., 2003) together with an increased risk of subsequent hypertension, cardiovascular disease and glucose intolerance in the adult offspring (Barker, 1997). The mode of action of dexamethasone in placental growth inhibition has still not been determined (Babat et al., 2005).

The placenta plays a pivotal role in fetal growth. Failure of placental growth and development during early and mid-pregnancy is directly associated with decreased fetal growth during late pregnancy (Hay et al., 1997). Despite the fact that placental development requires the coordinated action of trophoblast proliferation and differentiation, little is known about the mitotic regulators that play key roles in synchronizing these events (Genbacev et al., 2000). In this study, we aimed to shed light on the possible roles of cell cycle related proteins and cell cycle inhibitors in normal and growth retarded rat placentas. Moreover it is still not known how coordination mechanisms of proliferation and differentiation are influenced by dexamethasone-induced IUGR in the placenta. Therefore we aimed to examine the spatial and temporal immunolocalization, mRNA and protein levels of proliferating cell nuclear antigen (PCNA), cyclin D3, p27 and p57 in normal and dexamethasone-induced IUGR rat placentas. In addition we compared apoptotic cell numbers at light and electron microscopic levels to investigate the effects of maternally administered dexamethasone.

## Materials and methods

### Animals

Female Wistar rats (*Rattus norvegicus*) weighing 250–300 g used for all experiments were maintained under standard conditions and exposed to 12/12 h light–dark cycles. After mating, the presence of sperm in the vaginal smear the following morning was designated as day 0 of pregnancy. On day 13 of pregnancy, rats were subcutaneously injected with a dose of 100 μg dexamethasone 21-acetate (Dexamethasone) (Sigma–Aldrich, St. Louis, MO, USA) in 0.1 ml 10% ethanol. The animals subsequently received daily injections of 200 μg/kg dexamethasone on days 14–19 of pregnancy. Control animals were injected with 0.9% saline solution subcutaneously on corresponding days of pregnancy. Eight 0.9% saline injected (control group) and eight dexamethasone injected (IUGR group) female rats were sacrified on day 20 of pregnancy. The experimental protocols were approved by the Animal Care and Usage Committee of Akdeniz University and were in accordance with the guidelines of the International Association for the Study of Pain.

### Tissue preparation

The placentas were dissected and fixed in 4% formaldehyde (100 ml 37% formaldehyde, 900 ml distilled water, pH 7) at room temperature approximately for 12 h for immunohistochemistry and TUNEL staining.

### Immunohistochemistry

Formalin-fixed paraffin-embedded samples were cut into 5 μm sections and placed on slides coated with poly-L-lysine. After deparaffinization, slides were boiled in citrate buffer (pH 6.0) for 10 min for antigen retrieval and cooled for 20 min at room temperature. Then, sections were immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Slides were then incubated in a humidified chamber with UltraV block (Labvision, Fremont, CA, USA) for 15 min at room temperature. Excess serum was drained and sections were incubated with primary antibodies PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-56) mouse monoclonal antibody at 1:1000 dilution; cyclin D3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-6283) mouse monoclonal antibody at 1:400 dilution; p27 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-1641) mouse monoclonal antibody at 1:200 dilution; p57 (sc-1040; Santa Cruz Biotechnology, Santa Cruz, CA, USA) rabbit polyclonal antibody at 1:200 dilution for 1 hour at room temperature in a humidified chamber. Isotype controls were performed by replacing the primary antibody with the appropriate serum or non-immune IgG in the same dilutions as the specific antibodies. The sections were washed three times for 5 min with phosphate buffered saline (PBS) and then incubated with biotinylated secondary antibody (K0609; HRP LSAB-2 system, DakoCytomation, Glostrup, Denmark) for 30 min then