Expression of the Growth Arrest Genes (GAS and GADD) Changes during Organogenesis in the Rat Fetus

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ABSTRACT Mammalian cells mount an active response to nutrient limitation by overexpressing the growth arrest specific (GAS) and the growth arrest and DNA damage (GADD) genes. During embryogenesis in rats, there are quantitative and temporal differences in GAS and GADD gene expression during the development of the placenta, heart and kidney. Genes associated with the inhibition of DNA synthesis (p53 and GAS1) were predominantly expressed during the early stages of development, whereas those genes associated with inhibition of protein synthesis [GADD153 (also known as CHOP-10 or Ddit3) and C/EBP-β] were more highly expressed during the later stages. The GADD45 gene was expressed throughout development. There were distinct periods of GAS3 and GAS6 gene expression during the development of the placenta, heart and kidneys, which is consistent with the proposed roles of these genes in cell interactions. These results show that there is a change in the expression of genes associated with the negative regulation of growth as the fetus develops. J. Nutr. 129: 1532–1536, 1999.

KEY words: • rats • pregnancy • fetal growth • growth arrest • apoptosis

Epidemiological studies of human populations have shown that poor growth in utero is an important factor that predisposes an individual to the later development of type-2 (non-insulin-dependent) diabetes mellitus and hypertension in adulthood (Barker and Osmond 1986, Hales and Barker 1992). A number of animal studies have shown that the early periods of fetal growth are particularly sensitive to the nutritional status of the mother (Davison and Dobbing 1996, Widdowson and McCance 1963). Once growth has been perturbed, the fetus never fully recovers and continues to grow more slowly. Furthermore, an animal undernourished in utero does not develop symmetrically. For example, feeding pregnant rats a low-protein diet produces a modest decrease in the birth weight of the pups, but as a proportion of the whole body weight, the kidneys are more severely affected than the heart (Rees et al. 1999). Protein deficiency also affects the ultrastructure of organs including the kidney, liver and pancreas (Desai et al. 1995, Snoeck et al. 1990).

Little is known about the mechanisms that cause this asymmetric growth and development. Rat embryos up to d 11.5 of gestation can be successfully cultured in vitro, with growth rates that are close to those found in vivo, in a simple medium containing essential nutrients and serum (New 1978). These experiments show that the fetus and its placenta are largely autonomous and do not require positive growth factors from the mother (Alsat et al. 1995). There is also evidence that cells derived from the fetus have negative controls that respond to the availability of nutrients in the medium and suppress growth. This is not a passive process; growth arrest causes specific increases in mRNAs that are normally only found at low levels in exponentially dividing cells. Despite extensive study of cell cultures, there is little information on the role of these genes during fetal development.

Among the genes upregulated in response to nutrient stress in cell cultures are the growth arrest and DNA damage (GADD) genes (Fornace et al. 1989) and the growth arrest specific (GAS) genes (Schneider et al. 1988). Their expression is sensitive to a variety of nutrients, including amino acids (Fleming et al. 1998, Marten et al. 1994), nucleotides (Linke et al. 1996) or glucose (Carlson et al. 1993). The GADD and GAS genes code for a diverse range of proteins with a variety of functions, including the suppression of DNA synthesis (Ruoaro et al. 1997, Smith et al. 1994), the inhibition of differentiation (Batchvarova et al. 1995) and the induction of apoptosis (Brancollini et al. 1995, Fabbretti et al. 1995, Goruppi et al. 1996). The response of some genes is rapid but transient, with expression reaching its maximum within 24 h, whereas others respond more slowly over several d (Fleming et al. 1998). Other stimuli, such as DNA damage or contact inhibition, also increase gene expression. There is evidence for the involvement of the tumor suppressor p53 (Linke et al. 1996) and the transcriptional activator C/EBP-β (Smith et al. 1994) in these processes.

Previous reports have shown that the GAS and GADD...
genes are expressed during the development of several tissues (Coccia et al. 1992, Manfioletti et al. 1993, Nakano et al. 1996). This observation suggests that these genes may play a part in the negative control of fetal growth. To characterize their expression during the development of the rat fetus, we have measured the levels of the GAS and GADD gene mRNAs in the placenta, heart and kidneys. These three structures are formed at different stages in development: the placenta is the earliest to appear followed by the heart and finally the kidneys (Wilson and Warkany 1949). Our data show that the GAS and GADD genes are not expressed uniformly during the formation of these three organs. This change in the negative controls during development may be a possible mechanism mediating the effects of maternal under-nutrition on fetal development.

METHODS

Experimental animals. Female rats from the Rowett Hooded Lister strain were fed normal stock CRM diet (Proximate analysis: protein, 17.7%; crude oil, 2.4%; carbohydrate, 57%; fiber, 3.6%; digestible energy, 13.3 MJ/kg. Supplied by SDS, Witham, Essex, UK). Animals of ~230 g body weight were caged overnight with normal males, and mating was confirmed on the following morning by the presence of a plug (d 0.5). Rats were killed at different points during pregnancy; the placentas were separated from the fetuses, which were dissected; and the organs were frozen immediately in liquid nitrogen. All experimental procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

RNA extraction. Frozen tissue samples were transferred directly to Tri-reagent (Sigma, Poole, Dorset, UK) using ~1 mL per 100 mg of tissue. The samples were then disrupted by homogenization for 10 s to Tri-reagent (Sigma, Poole, Dorset, UK) using maximum speed (24,000 rpm). RNA was prepared from this mixture according to the manufacturers instructions.

cDNA probes. Probe templates for GADD153 were prepared from the plasmid pASA4 (Fornace et al. 1989) and for C/EBP-β from MSV/EBP-β (Yeh et al. 1995). Probes for the p53, GADD45, GAS1, GAS2, GAS3, GADD45, GAS5 and GAS6 genes were prepared by polymerase chain reaction (PCR) amplification of cDNA prepared from growth-arrested 3T3 cells (Fleming et al. 1998). The PCR primers used were described previously (Fleming et al. 1997).

Northern analysis. 20 µg of total RNA was separated on a 1.2% agarose gel. The gel was stained with ethidium bromide to confirm that equal amounts were loaded before transfer to a nylon membrane (Boehringer, Lewes, East Sussex, UK). Probe templates were labeled with [α-32P]-dCTP using a Megaprime labeling kit (Amersham International, Buckingham, UK) to give specific activities of 5 x 10⁹–5 x 10¹⁰ Bq/µg. Hybridizations were carried out according to standard protocols (Sambrook et al. 1989), and the blots were washed in 0.5 x 150 mmol NaCl/L · (15 mmol sodium citrate/L)¹ (SSC) + 1% SDS at 65°C. The distribution of radioactive activity was determined by using a wire proportional counter (Packard Instant Imager, Canberra-Packard, Pangbourne Bucks, UK) and by autoradiography. The relative expression of each mRNA was determined by measuring the amount of radioactive in each band on the Northern blot and correcting it for variations in loading and transfer efficiency by replotting the blots for 18S ribosomal RNAs. Where multiple transcripts were present, the radioactivity associated with both bands was assessed.

Statistical analysis. The RNA used for the blots shown in Figure 1, was derived from two separate placentas at each time point. The RNA in Figures 2 and 3 was extracted from the organs of four separate pups chosen at random. The duplicate sample was taken from a different set of four pups. Data shown in Figure 4 were analyzed by one-way ANOVA (Excel 5.0, Microsoft, Seattle, WA). Means for tissues were compared with the least significant difference test.

RESULTS

Preliminary experiments showed that the probe for GADD45 hybridized to two transcripts of 4.2 and 2.1 kb and GADD153 to two transcripts of 2.9 and 1.6 kb. In the GAS1 gene family, the GAS1 probe hybridized to a single transcript of 5.8 kb, GAS3 to two bands of 4.3 kb and 2.3 kb and GAS6 to a single transcript of 3.3 kb. A probe for mouse GAS5 failed to hybridize to rat RNA samples. The expression of GAS2 was confined to the limbs (data not shown). A probe for p53 hybridized to two transcripts of 5.9 and 2.6 kb and the probe for C/EBP-β to two transcripts of 2.6 and 1.9 kb. In genes with multiple mRNAs, there was some variation in the ratio of the transcripts; however, these differences were not reproducible. Previous data from tissue culture models (Fleming et al. 1998) indicates that the multiple mRNAs are regulated coordinately.

Figure 1 shows the changes in GAS and GADD expression during the development of the placenta from d 12, when the placenta is undergoing the final stages of growth, through to d 22. It should, however, be noted that the sample for d 12 was a complete conceptus, including the fetus. Although the bulk of the mRNA was derived from the placenta, which dominates at that stage, high levels of expression in the fetus cannot be ruled out. The p53, GADD45 and GAS6 genes showed their highest expression on d 12 of gestation. Thereafter, the mRNA levels fell, although all the genes continued to be expressed at a lower level. After placental growth slowed and ceased at ~d 19, the expression of the GADD153 and
C/EBP-β genes increased. The levels of GAS1 and GAS3 mRNA in the placenta were below the detection limit.

The pattern of gene expression in the heart from d 18 until to 2 d after birth is shown in Figure 2. By d 17 of gestation, the heart is almost completely formed, and there are no further changes in gross morphology until birth. The p53, GAS1, GADD45 and GAS3 genes all showed the highest levels of expression on gestational d 18. Expression then declined as gestation progressed. The fall in p53 and GAS3 mRNA levels was more pronounced than that of GAS1 and GADD45. The expression of GADD153 increased slightly, reaching a maximum by d 2 after birth. The expression of C/EBP-β, which was low during gestation, showed a marked increase after birth. The pattern of GAS6 expression in the heart was very different from that found in the placenta and kidney. It was expressed at a relatively constant level from gestational d 18 onwards, with only a slight drop on d 1 after birth.

The kidneys develop somewhat later than the other organs.

Wilson & Warkany (1949) showed that the metanephroi first appear on d 13, the first glomeruli are not present until d 18 and that the final formation of the nephrons is not complete until after birth. Growth arrest gene expression shown in Figure 3, can be divided into three periods. Early in the formation of the kidney, the p53, GAS1 and GAS3 genes were expressed. Between d 19 and 21 there was a second phase characterized by a peak in the expression of GAS6, and finally, just after birth, there was a considerable increase in the expression of C/EBP-β. The GADD45 gene was expressed throughout kidney development, with a small increase during the late stages of gestation and just after birth.

To examine the relative expression of these genes during development, a series of samples were prepared from fetuses on d 19 of gestation (Fig. 4). For simplicity the genes have been divided into three groups on the basis of their reported responses to different stresses. (Linke et al. 1996, Wang et al. 1996) The first group are associated with the inhibition of DNA synthesis and include the p53, GAS1 and GADD45 genes. The expression of p53 was highest in the kidneys and lowest in the placenta, with...
intermediate levels in the heart. The levels of GAS1 in the kidneys were significantly higher than those in the heart and placenta. The expression of GADD45 was lower in the heart than in placenta and kidney, although the differences were not as marked as those seen for p53 and GAS1.

The second group of genes, the GADD153 gene and the related bZip transcriptional activator C/EBP-β are associated with the inhibition of protein synthesis. (Wang et al. 1996) Expression of the GADD153 was widespread, and there were no significant differences among the three tissues. The expression of C/EBP-β was significantly higher in the placenta than in the heart or kidney.

The third group, the products of the GAS3 and GAS6 genes, are associated with the cell surface, the control of apoptosis and cell adhesion. Expression of GAS3 was highest in the kidney, intermediate in the heart and lowest in the placenta. The expression of GAS6 was significantly higher in the heart, with lower levels in the kidney and placenta.

**DISCUSSION**

It has been suggested that developmentally advanced tissues grow preferentially when the fetus is deprived of nutrients because their development has passed a point where they are no longer subject to certain growth controls (Barker 1994). These results show a change in the pattern of the nutrient-sensitive GAS and GADD gene expression as development proceeds. As a generalization, undifferentiated cells express higher levels of p53 and GAS1 and lower levels of GADD153 and C/EBP-β. There is a fall in p53 and GAS1 and an increase in GADD153 and C/EBP-β expression as the cells become terminally differentiated. At any given stage in development there are significant differences in GAS and GADD gene expression between different organs because they are at different stages of development. For example, the kidney, which is less advanced than the heart on d 19, expresses significantly higher levels of p53 and GAS1. Low levels of the mRNAs for these two genes are found in the placenta, which has developed completely at this stage. The p53 protein mediates reversible growth arrest in nucleotide deprived cells (Linke et al. 1996), so if the mRNA levels reflect an increase in activity, the sensitivity will decrease in the order kidney, heart and placenta. Conversely, because the placenta has the highest levels of C/EBP-β, which is induced by amino acid deficiency (Fleming et al. 1998), it will be more sensitive to growth arrest mediated through this route.

There is toxicological evidence to support this hypothesis. The inhibitor of nucleotide synthesis, phosphonacetyl l-aspartate acid, induces apoptosis through a p53-dependent mechanism and is embryotoxic when administered to pregnant mice. The fetus is 20 times more sensitive during the first half of pregnancy (Sieber et al. 1980a and 1980b), showing that the early stages of development are more sensitive to an interruption of the nucleotide supply. This suggests that the increased levels of p53 mRNA are associated with the presence of more functional protein. Accurate DNA replication is of paramount importance because defects or errors introduced at an early stage and left uncorrected would be propagated through whole populations of cells. It was suggested that high levels of the p53 gene in early embryonic cells serve to protect the nutrient-stressed fetus by excluding these damaged cells (Aladjem et al. 1998).

In fibroblasts, the p53 protein interacts directly with the GAS1 gene product, a plasma membrane glycoprotein that blocks the G_{2} to S phase transition (Del Sal et al. 1995). The biological function and mechanism of action of GAS1 are not yet understood. It was suggested that GAS1 may interact with integrins and modify the attachment of cells to the extracellular matrix (Evdokiou and Cowled 1998). Because GAS1 is expressed early during the development of both the heart and kidney, it may be an important link between the nucleotide supply and organ morphology. Although the GADD45 gene is also regulated by p53, other factors, including C/EBP-α, control its expression in terminally differentiated cells (Constance et al. 1996). The GADD45 gene product binds to the proliferating cell nuclear antigen, blocks replication and allows DNA damage to be repaired before the cells enter into the S phase (Smith et al. 1994). Because GADD45 expression is largely independent of cell phenotype, it may be a useful marker of a decrease in the available nucleotide supply or DNA damage at any stage of gestation.

The increase in GADD153 and C/EBP-β expression during the later stages of fetal development may be a response to the increasing metabolic demands as protein accretion approaches its maximum during the latter stages of gestation. The C/EBP transcriptional activators were shown to play an important part in the direct and indirect regulation of growth. Directly, they regulate the normal differentiation of cells, particularly those with extensive metabolic functions, such as adipocytes and hepatocytes (Birkenmeier et al. 1989, Yeh et al. 1993). Indirectly, C/EBP transcriptional activators may regulate the local production of growth factors. The genes for insulin-like growth factor (IGF)-I and IGF-II, growth hormone, leptin and various cytokines all have C/EBP-binding sites in their promoters (Miller et al. 1996, Nolten et al. 1994, O’Brien et al. 1994, Schaefe 1996, van Dijk et al. 1992). Fetal growth may be inhibited by a reduced production of these and other
growth factors and implies a shift towards a more indirect regulation of growth during the latter stages of gestation.

The remaining growth arrest genes, GAS3 and GAS6, appear to regulate differently (Fleming et al. 1998), and their expression may be associated with specific cell interactions (Antipatis et al. 1998). The protein products have very different functions: GAS3 is involved in the induction of apoptosis (Fabbretti et al. 1995), whereas the GAS6 gene product regulates cell adhesion as well as being a protein tyrosine kinase ligand (McCloskey et al. 1997). By regulating adhesion and apoptosis, these genes have an important role in the cell interactions that shape tissue morphology. Both genes are expressed during the development of the heart and kidney, and during these periods specific, structures within the organs may be particularly sensitive to nutrient deficiency.

The present results show that genes from the GAS and GADD families play a role in normal fetal development. Because these genes are not expressed uniformly in all fetal tissues, the response to nutrition may vary among different organs. Also, because their mRNA levels can be regulated in response to the nutrient supply, the specific timing of their expression may also define windows when particular organs are susceptible to the negative control of growth. This may produce asymmetrical fetal growth and development, which permanently changes the subsequent physiology of the adult.

LITERATURE CITED


