

Protein levels of genes encoded on chromosome 21 in fetal Down syndrome brain: Challenging the gene dosage effect hypothesis (Part IV)

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Received November 28, 2002

Accepted March 10, 2003

Summary. Down syndrome (DS) is the most frequent genetic disorder with mental retardation and caused by trisomy 21. Although the molecular mechanisms of the various phenotypes of DS could be due to overexpression of gene(s) on chromosome 21, several groups have challenged this gene dosage effect hypothesis. The near completion of the sequencing of human chromosome 21 provides unprecedented opportunities to understand the molecular pathology of DS, however, functional information on gene products is limited so far. We therefore evaluated the levels of six proteins whose genes are encoded on chromosome 21 (trefoil factor 1, trefoil factor 2, trefoil factor 3, coxsackie virus and adenovirus receptor, carbonyl reductase 1 and interferon- α receptor) in fetal cerebral cortex from DS and controls at the early second trimester using Western blot analysis. None of the investigated proteins showed overexpression in DS compared to controls suggesting that these proteins are not involved in abnormal development of fetal DS brain and that DS phenotype can not be simply explained by the gene dosage effect hypothesis. We are systematically quantifying all proteins whose genes are encoded on chromosome 21 and these studies may provide a better understanding of genotype-phenotype correlation in DS.

Keywords: Carbonyl reductase 1 – Chromosome 21 – Coxsackievirus and adenovirus receptor – Down syndrome – Interferon- α receptor – Trefoil factor

Abbreviations: AD, Alzheimer's disease; CAR, coxsackievirus and adenovirus receptor; CBR1, carbonyl reductase 1; CNS, central nervous system; DS, Down syndrome; IFNs, interferons; IFNAR-1, interferon- α receptor; NSE, neuron specific enolase; TFF, trefoil factor

Introduction

Down syndrome (DS), trisomy 21, is the most common genetic cause of mental retardation with a prevalence of 1:700 live births. Individuals with DS show a series of neuropathological features, including reduction of brain size, abnormal neuronal migration, differentiation and abnormal dendritic arborization. The presence of an extra copy of genes on a chromosome 21 segment is predicted

to contribute to aspects of the complex DS phenotype. It is often assumed that all genes on chromosome 21 will be overexpressed at a level 1.5 fold relative to diploids. However, since regulation of gene expression is a complex mechanism, this prediction cannot be longer sustained (Marks et al., 1996; Greber-Platzter et al., 1999; Engidawork et al., 2001; Gulesserian et al., 2001).

The virtually complete sequencing of chromosome 21 provides the opportunity to test, which genes are involved in DS, and how individual genes contribute to the overall phenotype. The study of gene expression patterns is now of crucial importance as an essential step towards understanding gene function. Although a large series of proteins have been found to be deranged in adult DS brain (which maybe however confounded by Alzheimer's disease (AD) from the fourth decade), information on chromosome 21-proteins in developing DS brain is limited so far. This made us investigate expression levels of six proteins encoded on chromosome 21: trefoil factor 1–trefoil factor 3 (TFF1–TFF3; 21q22.3), coxsackievirus and adenovirus receptor (CAR; 21q21.1), carbonyl reductase 1 (CBR1; 21q22.13) and interferon- α receptor (IFNAR-1; 21q22.11) in fetal brains from DS and controls (Fig. 1).

The “TFF domain” is an ancient cysteine-rich shuffled module forming the basic unit for the family of secretory TFF peptides (formerly P-domain peptides and trefoil factors). It is also an integral component of mosaic proteins associated with mucous surfaces (Bork, 1993). Three mammalian TFF peptides are known (i.e., TFF1–TFF3) and they were originally detected as typical secretory products of a variety of gut and mucous epithelia. TFF peptides are also found in the central nervous system (CNS), in mammals

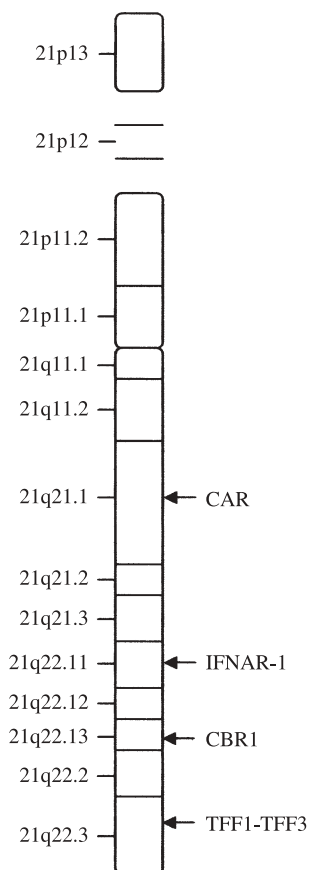


Fig. 1. Giemsa banding (G-bands) of human chromosome 21. Arrows indicate six gene products encoded on chromosome 21, examined in this study

and only minor amounts are detectable in the brain (see review by Hoffmann and Jagla, 2002).

CAR is a 46 kDa cell surface protein that mediates viral attachment and infection and it belongs to the immunoglobulin superfamily (Bergelson et al., 1997). Although considerable progress towards the characterization of CAR has been made, the cellular and physiological function of CAR still remains to be discovered. CBR1 is a monomeric NADPH-dependent enzyme that reduces a large number of biologically and pharmacologically active carbonyl compounds to their corresponding alcohols (Wermuth, 1985). CBR1 has been shown to function as both, an aldo-keto reductase and a quinone reductase (Wermuth, 1982). The exact roles of CBR1 in cellular metabolism are still elusive, however, the ability of CBR1 to metabolise reactive carbonyls and quinone suggests that it may play a role in the modulation of oxygen free radicals with the potential for DNA damage and increased mutagenesis. Interferons (IFNs) are members of the cytokine family that elicit antiviral, antiproliferative, and immune modulatory effects (Pestka et al., 1987). The type I IFN receptor (IFNAR) consists of two

subunits, IFNAR-1 and IFNAR-2. Human IFNAR-2 has moderate intrinsic affinity for the range of IFNs, whereas its partner, human IFNAR-1, alone binds IFNs weakly. However, IFNAR-1 plays an essential role by contributing to both, the final high affinity and the differential specificity of the IFNAR complex (Cutrone and Langer, 1992).

Materials and methods

Fetal brain samples

Fetal brain tissues (cerebral cortex) of DS (8 females with 19.44 ± 1.12 weeks of gestational age) and controls (6 females with 19.17 ± 1.60 weeks of gestational age) were used in this study. Brain samples were obtained from Dr. Mara Dierssen (Medical and Molecular Genetics Center-IRO, Hospital Duran i Reynals, Barcelona, Spain) and Dr. Joan Carles Ferreres (Department of Pathology UDIAT-CD, Corporació Sanitària Parc Tauli, Sabadell, Barcelona, Spain). All samples had a post-mortem time of less than 6 hours, and were stored at -70°C with a freezing chain never interrupted until use.

Antibodies

The details of the preparation and characterization of antibodies have been described previously: TFF1–TFF3 (rabbit polyclonal antibodies, Wiede et al., 1999; Jagla et al., 2000); CAR (rabbit antiserum, Cohen et al., 2001); CBR1 (rabbit antiserum, Ishikura et al., 1998); IFNAR-1 (mouse monoclonal antibody (IgG1), Ling et al., 1995; Goldman et al., 1999). Two antibodies for neuron specific enolase (NSE; Chemicon, UK) and actin (Sigma, USA), were purchased.

Western blotting

Fetal brain tissues ground under liquid nitrogen were homogenized in lysis buffer containing protease inhibitor cocktail tablets (Roche, Germany) at 4°C and centrifuged at $8,000 \times g$ for 10 minutes. The BCA protein assay kit (Pierce, USA) was applied to determine the concentration of protein in the supernatant. Samples ($10 \mu\text{g}$) were mixed with the sample buffer (100 mM Tris-HCl, 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 15 minutes and loaded onto an ExcelGel SDS homogenous gel (Amersham Pharmacia Biotech, Sweden). Electrophoresis was performed with Multiphor II Electrophoresis System (Amersham Pharmacia Biotech). Proteins separated on the gel were transferred onto PVDF membrane (Millipore, USA) and membranes were incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 2% non-fat dry milk). Membranes were incubated for 2 hours at room temperature with diluted primary antibodies (1:1,000 for TFF1–TFF3, CBR1, IFNAR-1 and actin; 1:2,000 for CAR; 1:3,000 for NSE). After 3 times washing for 15 minutes with blocking buffer, membranes were probed with secondary antibodies (goat anti-rabbit IgG(H + L) for TFF1–TFF3, CAR, and CBR1 and goat anti-mouse IgG1 for IFNAR-1) coupled to horseradish peroxidase (Southern Biotechnology Associates, Inc., USA) for 1 hour. Membranes were washed 3 times for 15 minutes and developed with the Western Lightning™ chemiluminescence reagents (PerkinElmer Life Sciences, Inc., USA).

Statistics

The density of immunoreactive bands was measured by RFLPscan version 2.1 software program (Scanalytics, USA). Between group differences were calculated by non-parametric Mann-Whitney U test using GraphPad Instat2 program and the level of significance was considered at $P < 0.05$.

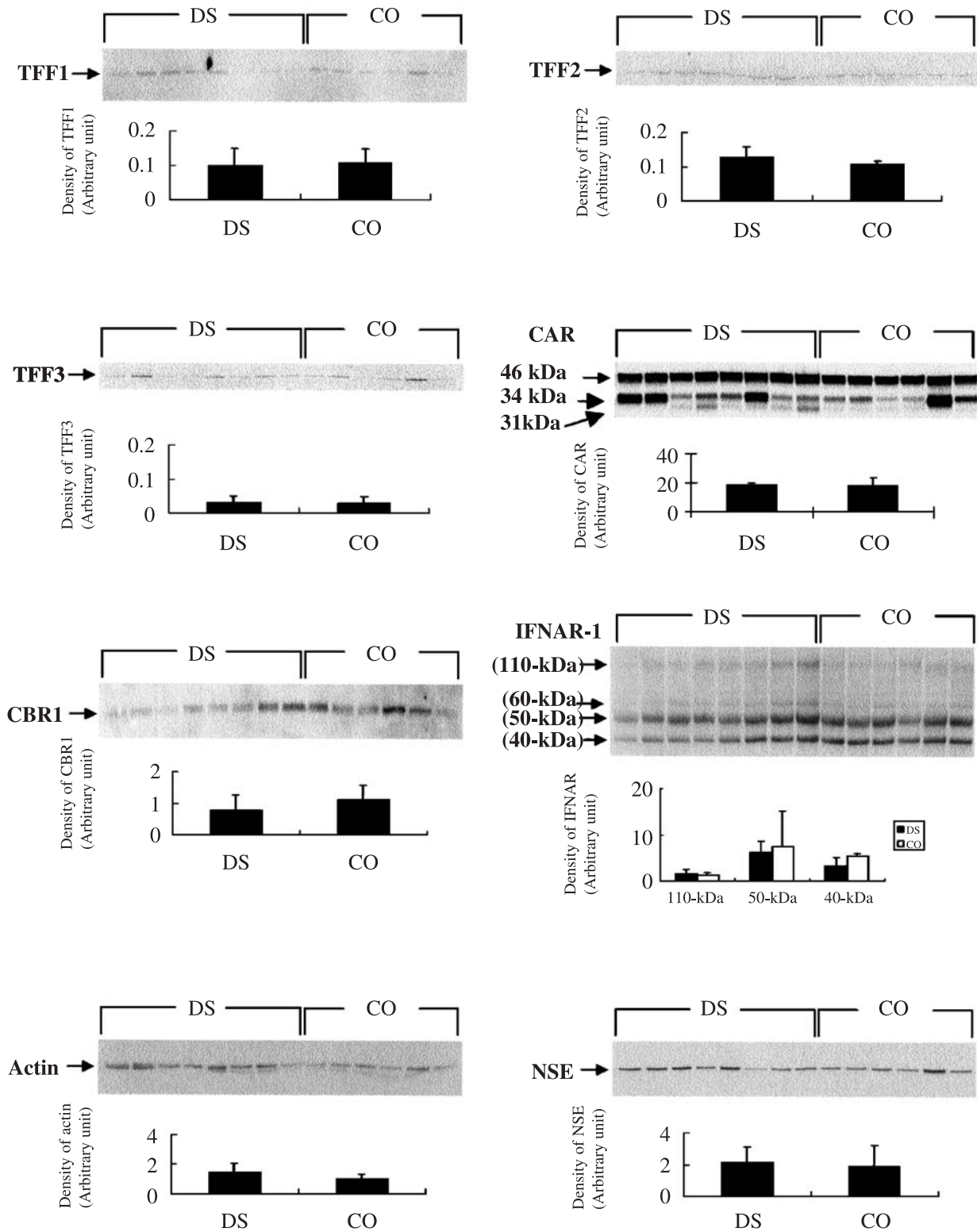


Fig. 2. Western blot analysis for six proteins whose proteins are encoded on chromosome 21 in cerebral cortex from fetal brain with DS and controls. Denatured proteins (10 μ g) were loaded, separated on a homogeneous gel and transferred onto PVDF membrane. As described in 'materials and methods', the membranes were incubated with primary and secondary antibodies, and immunoreactive bands (TFF1, 19 kDa; TFF2, 28 kDa; TFF3, 28 kDa; CAR, 46 kDa; CBR1 30 kDa; IFNAR-1, 40, 50, 60 and 110 kDa; NSE, 45 kDa; actin, 42 kDa) were detected using chemiluminescence reagents. The density of detected bands was measured and calculated by non-parametric Mann-Whitney U test, and the level of significance was considered at $P < 0.05$

Result

We evaluated the expression level of six proteins (Fig. 1) encoded on chromosome 21 (TFF1–TFF3, CAR, CBR1 and IFNAR-1) in fetal brains with DS compared to controls by Western blot analysis. As shown in Fig. 2, the density of immunoreactive bands of eight proteins investigated in this study was comparable between DS and controls ($p > 0.05$). We detected one band at 46 kDa and doublet at 34–31 kDa with CAR antiserum. The anti-IFNAR-1 antibody recognized two strong bands at 40 and 50 kDa and two weak bands at 60 and 110 kDa. Two

proteins, NSE and actin were used as reference proteins for neurons, and total cells, respectively, and showed comparable expression levels in DS and controls, except for actin-normalized protein at 40 kDa of IFNAR-1 showing a decreased level in DS (Fig. 3).

Discussion

TFF1 is widely distributed throughout the adult rat brain, with pronounced expression in the hippocampus, frontal cortex, and the cerebellum. A developmentally regulated expression was reported for TFF1 in the rat. Expression of

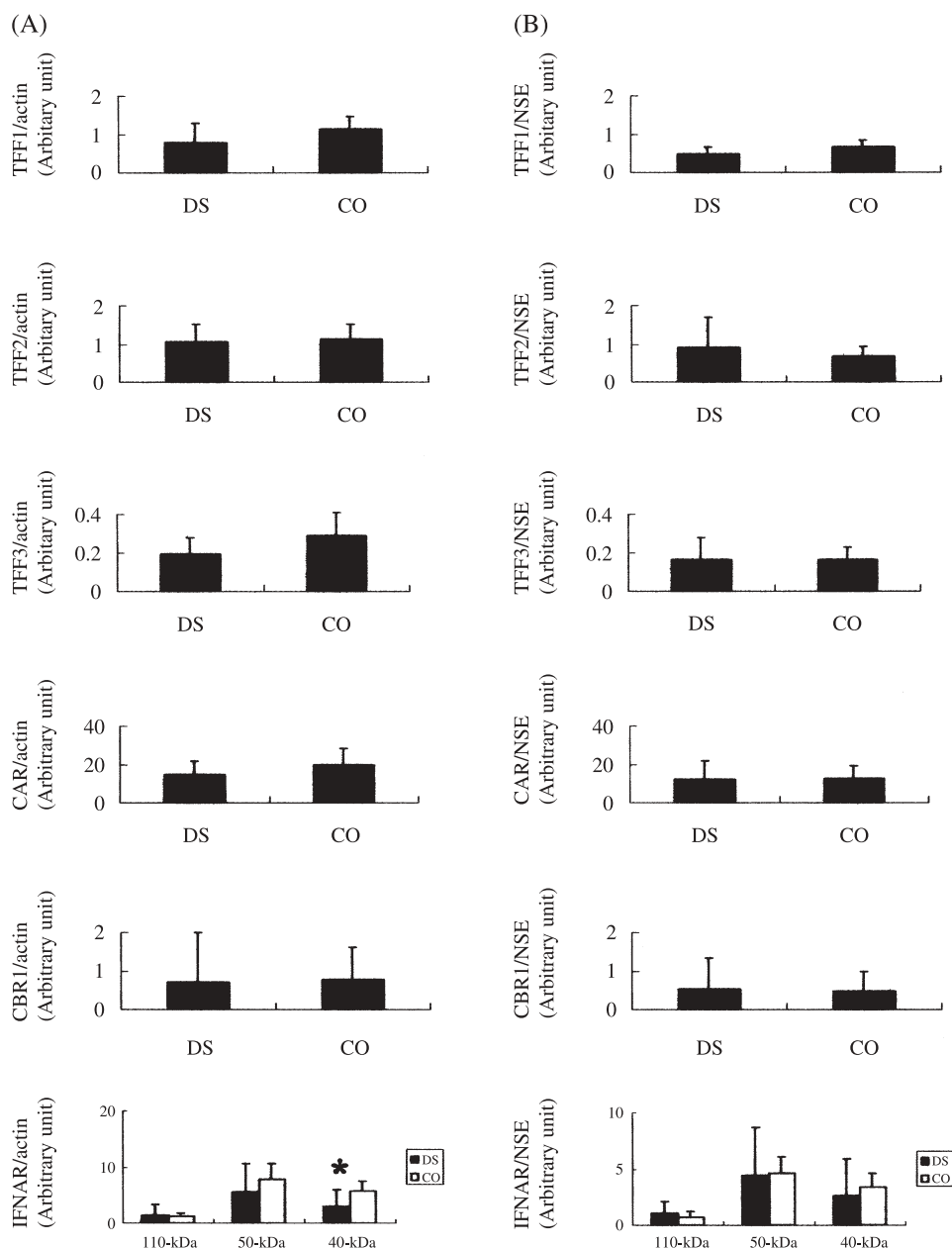


Fig. 3. Expression levels of six proteins normalised with those of actin (A) or NSE (B). The density of immunoreactive band for each protein was normalized with that of actin or NSE, which were used as reference proteins. * $p < 0.05$

TFF1 mRNA in the hippocampus reached a maximum around birth when maturation occurs and then gradually decreased (Hitora et al., 1995). This led to the hypothesis that TFF1 may play an important role during brain development. It was shown that human TFF2 is *N*-glycosylated, in contrast to porcine, mouse or rat TFF2 (May et al., 2000), but there is no report on neural expression of TFF2. TFF3 is a neuropeptide synthesized in the hypothalamus of rat and human (Probst et al., 1995). Oxytocinergic neurons in the human supraoptic and paraventricular nuclei were shown to be the sole site of synthesis. These neurons project to the posterior pituitary, in which relatively large amounts of TFF3 are stored awaiting its release into the bloodstream (Jagla et al., 2000). Remarkably, the molecular weight of TFF3 from the posterior pituitary appeared somewhat larger than that from the intestine. The molecular mass of bands for all three TFF peptides detected in this study was also higher than expected. At the moment it is difficult to explain this result, but since TFF3 dimerisation has been reported (Kinoshita et al., 2000), it could be due to this fact or to posttranslational modifications in brain.

All TFF peptides have been shown to enhance cell migration *in vitro* (motogenic effect; Hoffmann et al., 2001). The regulation of cell motility by TFF peptides implies that TFF peptides have a major influence on restructuring of the cytoskeleton. In our experiments, expression levels of all three TFF peptides were similar in fetal DS and normal brain and these results suggest that TFF peptides may not be responsible for cytoskeleton derangement (Gulesserian et al., 2002) or deficiency of neuronal migration and dendritic outgrowth in fetal DS (Weitzdoerfer et al., 2001). In addition to motogenic effect, inhibition of apoptosis was reported for TFF2 (Lalani et al., 1999) and TFF3 (Chen et al., 2000). These results obtained *in vitro* are consistent with *in vivo* observation that transgenic TFF3 (*-/-*) mice have increased intestinal apoptosis (Taupin et al., 2000). Taken together with comparable expression of PEP19, α A-crystallin and cystatin B, whose genes are also encoded on chromosome 21 and have the ability to protect cells from apoptosis (Cheon et al., 2003a, b), unaltered expression of TFF peptides in fetal DS brain suggests that enhanced apoptosis may not be involved in the abnormal brain development of DS.

With CAR antiserum, we detected one protein band at 46 kDa, the size expected for full-length CAR, as well as a doublet of variable intensity at 34–31 kDa. The lower molecular weight bands have been observed previously in CAR immunoblots (Xu et al., 1995; Xu and Crowell, 1996), and may represent alternative forms of the receptor. Alternatively spliced and truncated CAR mRNAs have been identified by other investigators (Bergelson et al., 1998; Fechner et al., 1999; Thoelen et al., 2001), and a truncated form of

CAR protein has been observed in malignant pleural effusions (Bernal et al., 2002). Recent reports indicated that CAR functions in homophilic adhesion and the formation of intracellular junctions (Honda et al., 2000; Cohen et al., 2001). CAR is expressed abundantly in the embryonic mouse brain, and its expression declines rapidly after birth, suggesting that it may function in organization of the developing brain (Xu and Crowell, 1996; Honda et al., 2000). However, the finding that CAR was expressed equally in fetal DS and normal brain tissue does not support a role for CAR overexpression in the pathogenesis of DS.

CBR1 was found in all human tissues tested and the highest expression was found in neuronal and glial cells of CNS (Wirth and Wermuth, 1992). Controversially, CBR1 mRNA was increased in lymphoblasts from DS patients (Lemieux et al., 1993), but there was no significant increase in transcript for CBR1 in myeloblasts from children with DS (Taub et al., 1999). Recently, we observed the increased expression of CBR1 protein in adult brain from DS and AD (Balcz et al., 2001), but this simply may reflect increased carbonyls in neurodegeneration. Herein, however, CBR1 was comparable between fetal DS brain and controls and this finding is in agreement with our previous study showing unchanged Cu/Zn superoxide dismutase (SOD-1) in fetal DS brain (Gulesserian et al., 2001). Unaltered SOD-1 may produce physiological levels of hydrogen peroxide, that is responsible for the generation of reactive oxygen species and reactive carbonyls and this in turn may not induce overexpression of their reducing enzyme, CBR1. Therefore, comparable expression of CBR1 does not explain increased reactive oxygen species formation in primary cultures of DS fetal neurons (Busciglio and Yankner, 1995) and elevated parameters that are known to be involved in the cellular oxidation in DS fetal brain (Odetti et al., 1998).

Binding of IFNAR to the type I IFNs triggers tyrosine phosphorylation of a number of proteins including JAK1, TYK2, STAT proteins and IFNAR-1 and IFNAR-2 subunits themselves to activate down-stream signal cascades (Velazquez et al., 1992). Particularly, TYK2 was shown to interact with IFNAR-1 (Colamonici et al., 1994) and mutant cells lacking TYK2 have significantly reduced IFNAR-1 protein level (Gauzzi et al., 1997). It was revealed that IFNAR-1 protein is localized in microglial cells and constitutively expressed in human brain. Moreover, the immunostaining of IFNAR-1 was increased in microglia of AD brains and in macrophages of cerebral infarct areas suggesting some roles of the IFNs-IFNAR system in these pathological processes (Yamada and Yamanaka, 1995). The cDNA for human IFNAR-1 encodes a 557 amino acids protein with 12 putative *N*-linked glycosylation sites. The protein itself has

predicted size of 64 kDa. In this study, however, we detected multiple bands and none of them was changed in fetal DS brain. Several reports have also demonstrated higher molecular weight ranging from 100 to 150 kDa as well as diffuse bands due to heavy glycosylation (Ling et al., 1995; Goldman et al., 1999). Two bands with lower molecular masses could be explained by the identification of mRNAs encoding two different soluble forms, lacking the transmembrane domain of IFNAR-1 in human cells (Abramovich et al., 1994) and by detection of soluble IFNAR-1 protein in human body fluids (Novick et al., 1992). Moreover, in agreement with present results, five bands in immunoblots of human brain proteins were detected including one of 110 kDa which may be IFNAR-1, one of 75 kDa may be a cross-linked product and two bands at 40 and 50 kDa may represent soluble receptor proteins (Yamada and Yamanaka, 1995). Comparable expression of IFNAR-1 between fetal DS brain and controls suggests that the type I IFNs-related signal pathway functions normally in fetal DS brain, although there is no report showing function and expression of TYK2 or STAT in DS.

Our present data showing unaltered expression of six proteins of chromosome 21 in fetal DS brain suggest that these may not be involved in the abnormal development of fetal DS brain at the early second trimester and that the DS phenotype is not simply explained by the gene dosage effect hypothesis. We are continuing to quantify proteins whose genes are encoded on chromosome 21 and these studies may advance our knowledge on gene function and regulation by providing expression profiles of disease related gene products.

Acknowledgements

We are highly indebted to the Red Bull Company (Salzburg, Austria) for generous support of the study. We thank Dr. Werner Hoffmann (anti-TFF1-TFF3 antibodies; Institut fuer Molekularbiologie und Medizinische Chemie, Otto-von-Guericke Universitaet, Magdeburg, Germany), Dr. Jeffrey M. Bergelson (anti-CAR antiserum; Division of Immunologic and Infectious Diseases, The Children's of Hospital of Philadelphia, PA, (USA) and Biogen, Inc. (anti-IFNAR-1 antibody; Cambridge, USA) for kindly providing the antibodies and comments.

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